Improved phytases

Phytases are enzymes that hydrolyze phytate (myo-inositol hexakisphosphate) to myo-inositol and inorganic phosphate. They
5 are known to be valuable feed additives.

The present invention relates to improved phytases, viz. phytases of amended characteristics, e.g. amended activity characteristics, reference being made to e.g. the phytase(s) it has been derived from, or to known phytases. Amended activity
10 characteristics means amended in at least one phytase activity related respect, such as (non-exclusive list): pH stability, temperature stability, pH profile, temperature profile, specific activity (in particular in relation to pH and temperature), substrate specificity, substrate cleavage pattern, substrate
15 binding, position specificity, the velocity and level of release of phosphate from corn, reaction rate, phytate degradation rate), end level of released phosphate reached.

Examples of amended activity characteristics are amended specific activity (e.g. increased, e.g. increased at a pH of 3,
20 4, 5, or 6); amended pH or temperature profile; and/or amended (e.g. increased) thermostability, e.g. of an increased melting temperature as measured using Differential Scanning Calorimetry (DSC).

The present invention also relates to a process for the
25 preparation of a modified protein, wherein in a first step a consensus sequence is determined from a number of highly homologous sequences according to steps a), b) and c) below:

a) at least three, preferably at least four amino acid sequences are aligned by any standard alignment program known in
30 the art;

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b) at every position of the amino acid sequence alignment, the amino acids are evaluated for their evolutionary similarity and a consensus residue is chosen by any standard program known in the art, whereby the minimal requirements for calculation of 5 a consensus residue are set in such a way that the program is already able to determine a consensus residue if a given residue occurs in only two of the aligned sequences. However, if there is a subgroup of sequences among the compared amino acid sequences that shows a much higher degree of similarity with 10 each other than with the remaining sequences of the alignment, the subgroup may be represented in the calculation only with its consensus sequence determined in the same way as outlined in EP 897985, or alternatively, to each sequence of the subgroup, a vote weight of 1 divided by the number of sequences in the 15 subgroup will be assigned;

c) in case no consensus amino acid at a defined position is identified by the program, any of the amino acids, preferably the most frequently occurring amino acid at this position is selected.

20 In a second aspect of the invention, a homologous sequence is compared with the consensus sequence, and one or more non-consensus residues in this homologous sequence are replaced by the corresponding consensus residues.

Preferably, only such amino acid residues are replaced in 25 the homologous amino acid sequence where a consensus residue can clearly be defined by the program under moderately stringent conditions whereas at all positions of the alignment where no preferred consensus amino acid can be determined under moderately stringent conditions, the amino acids of the 30 homologous protein remain unchanged.

In a third aspect of the invention, the active center of the protein of interest is determined, comprising all amino acid residues that are involved in forming the active center, both in the consensus sequence, and in the sequence of a homologous

5 protein; subsequently, some or all of the divergent amino acid residues of the homologous protein are inserted in the backbone of the consensus sequence.

In one embodiment of this process, the program used for the comparison of amino acids at a defined position regarding

10 their evolutionary similarity is the program "PRETTY".

The active center of the protein can be determined by using an analysis of the three-dimensional structure of the protein.

An example of a homologous protein is an enzyme family, an

15 example of a defined protein family is the family of phytases, e.g. of fungal origin.

For example, the amino acid sequence of the phytase can be changed by the introduction of at least one mutation or substitution chosen from

E58A	F54Y
D69K	I73V
D197N	K94A
T214L	R101A
E222T	N153K
E267D	V158I
R291I	A203G
R329H	S205G
S364T	V217A
A379K	A227V
G404A	V234L
	P238A

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Q277E

A287H

A292Q

V366I

A396S

E415Q

G437A

R451E

For interpreting these abbreviations, as an example, the mutation E58A is to be interpreted as follows: When subtracting 26 from the number, you get the position or residue number in the consensus phytase sequence or another phytase sequence aligned as shown in Fig. 1 (corresponding to the addition of a 26 amino acid signal sequence to the sequences shown in Fig. 1). For example, in E58A, number 58 means position number 32 (58-26=32). And the letter before the number, i.e. E, represents the amino acid in the phytase to be modified which is replaced by the amino acid behind the number, i.e. A.

The above-mentioned amino acid replacements, alone and/or in combination, have a positive effect on the protein stability.

The following sub-groups of mutations are also interesting (i.e. phytases comprising at least one mutation selected from either one of the groups of):

E58A, D69K, D197N, T214L, E222T, E267D, R291I, R329H, S364T, A379K, G404A;

F54Y, I73V, K94A, R101A, N153K, V158I, A203G, S205G, V217A, A227V, V234L, P238A, Q277E, A287H, A292Q, V366I, A396S, E415Q, G437A, R451E;

E58A, D69K, D197N, F54Y, I73V, K94A;

T214L, E222T, E267DR101A, N153K, V158I;

R291I, R329H, S364TA203G, S205G, V217A;

A379K, G404AA227V, V234L, P238A, Q277E;

A287H, A292Q, V366I, A396S, E415Q, G437A, R451E;

T214L, E222T, S364T, V158I, A203G, G404A, A227V, P238A, A396S, G437A, R451E.

5   Examples of host cells are plant cells, animal cells, and microbial cells, e.g. prokaryotic or eukaryotic cells, such as bacterial, fungal or yeast cells. An example of a fungal host is a strain of the genus Aspergillus, and examples of yeast hosts are strains of Saccharomyces, and strains of Hansenula.

10   The invention also relates to a modified protein obtainable or obtained by any of the processes described above.

The invention also relates to a variant or mutein of a phytase such as (but not limited to) the consensus phytase-1, wherein, in the amino acid sequence in Figure 2, at least one

15   of the following replacements have been effected: Q50L, Q50T, Q50G, Q50T-Y51N, Q50L-Y51N or Q50T-K91A.

In the third aspect mentioned above, a consensus sequence is determined from homologous sequences as described above; in a second step the active center of the protein comprising all

20   amino acid residues that are involved in forming the active center is determined in the consensus sequence and in the sequence of a single homologous protein as well. The single homologous protein may have preferred properties like high specific activity or different pH dependency of enzymatic

25   activity. In a third step some or all amino acid residues that are involved in forming the active center of the homologous protein are inserted into the backbone of the consensus sequence. The result thereof is a chimeric protein having the active center derived from a single protein and the backbone of

30   the consensus sequence.

The active center of the protein can be determined e.g. by using any analysis of the three-dimensional structure of the protein, e.g. by homology modelling on the basis of a known 3D-structure of a known protein.

5   The present invention also provides consensus proteins obtainable or obtained by such processes, in particular proteins comprising at least one of the amino acid sequences shown in Figures 2-6, 10 or 21, or variants or muteins thereof. Examples of such variants are shown in Figs. 7-9.

10   Such variants or muteins can be defined and prepared on the basis of the teachings given in European Patent Application number 0897010, e.g. Q50L, Q50T, Q50G, Q50L-Y51N, or Q50T-Y51N. These mutations are defined as above, or, alternatively, by reference to Fig. 2. When referring to Fig. 2, no subtraction of

15   the 26 amino acid signal peptide is required (e.g. in "Q50L," at position 50 of the amino acid sequence of Fig. 2, the amino acid Q has been replaced by amino acid L).

A food, feed, or pharmaceutical composition comprising the phytases of the invention is another aspect of the invention.

20   In this context, and relating to the process of the invention, "at least three, preferably at least four amino acid sequences of such defined protein family" means that three, four, five, six to twelve, twenty, fifty, or even more sequences can be used for the alignment and the comparison to create the

25   amino acid sequence of the consensus protein. "Sequences of a defined protein family" means that such sequences fold into a three-dimensional structure, wherein the alpha-helices, the beta-sheets and beta-turns are at the same position so that such structures are, as called by the man skilled in the art, largely

30   superimposable. Furthermore these sequences characterize proteins that show the same type of biological activity, e.g. a

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defined enzyme class, e.g. the phytases. The three-dimensional structure of one such protein is sufficient to allow the modelling of the structure of the other homologous proteins of such a family. An example, how this can be done, is given in
5   Example 1. "Evolutionary similarity" in the context of the present invention refers to a scheme which classifies amino acids regarding their structural similarity which allows that one amino acid can be replaced by another amino acid with a minimal influence on the overall structure, as this is done e.g.
10 by programs, like "PRETTY", known in the art. The phrase "the degree of similarity provided by such a program...is set to less stringent number" means in the context of the present invention that values for the parameters which determine the degree of similarity in the program used in the practice of the present
15 invention are chosen in a way to allow the program to define a consensus amino acid for a maximum of positions of the whole amino acid sequence, e. g. in case of the program PRETTY a value of 2 or 3 for the THRESHOLD and a value of 2 for the PLURALITY can be chosen. Furthermore, "a vote weight of one divided by the
20 number of such sequences" means in the context of the present invention that the sequences which define a group of sequences with a higher degree of similarity as the other sequences used for the determination of the consensus sequence only contribute to such determination with a factor which is equal to one
25 divided by the number of all sequences of this group.

As mentioned before, should the program not allow to select the consensus amino acid, the most frequent amino acid is selected; should the latter be impossible the man skilled in the art will select an amino acid from all the sequences used for
30 the comparison which is known in the art for its property to improve the thermostability in proteins as discussed e.g. by

Janecek, S. (1993), Process Biochem. 28, 435-445; Fersht, A. R. & Serrano, L. (1993), Curr. Opin. Struct. Biol. 3, 75-83; Alber, T. (1989), Annu. Rev. Biochem. 58, 765-798; Matthews, B. W. (1987), Biochemistry 26, 6885-6888; or Matthews, B. W. (1991), Curr. Opin. Struct. Biol. 1, 17-21.

The stability of an enzyme is relevant for many industrial applications. Therefore, a lot of attempts, more or less successful, have been made to improve the stability, preferably the thermostability of enzymes by rational or random approaches.

Here we present an alternative way to improve the thermostability of a protein.

The invention provides a process for the preparation of a consensus protein comprising a process to calculate an amino acid residue for nearly all positions of a so-called consensus protein and to synthesize a complete gene from this sequence that can be expressed in a pro- or eukaryotic expression system.

DNA sequences of the present invention can be constructed starting from genomic or cDNA sequences encoding the proteins, e.g. phytases, of interest. For example, they can be constructed by methods of in vitro mutagenesis [see e.g. Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory Press, New York]. A widely used strategy for "site-directed mutagenesis", as originally outlined by Hurchinson and Edgell [J. Virol. 8, 181 (1971)], involves the annealing of a synthetic oligonucleotide carrying the desired nucleotide substitution to a target region of a single-stranded DNA sequence wherein the mutation should be introduced [for review see Smith, Annu. Rev. Genet. 19, 423 (1985), and for improved methods, see references 2-6 in Stanssen et al., Nucl. Acids Res., 17, 4441-4454 (1989). Another possibility of mutating a given DNA sequence is the mutagenesis by using the polymerase chain reaction (PCR). DNA as

starting material can be isolated by methods known in the art and described e.g. in Sambrook et al. (Molecular Cloning) from the respective strains.

For strain information, see e.g. EP 684313 or any depository authority indicated below. Aspergillus niger [ATCC 9142], Myceliophthora thermophila [ATCC 48102], Talaromyces thermophilus [ATCC 20186] and Aspergillus fumigatus [ATCC 34625] have been redeposited according to the conditions of the Budapest Treaty at the American Type Culture Cell Collection under the following accession numbers: ATCC 74337, ATCC 74340, ATCC 74338 and ATCC 74339, respectively. It is, however, understood that DNA encoding a consensus protein in accordance with the present invention can also be prepared in a synthetic manner as described, e.g. in EP 747483 or EP 897985, or in the examples, by methods known in the art.

For sequence information, see e.g. EP 684313, or sequence data bases, for example like Genbank (Intelligenetics, California, USA), European Bioinformatics Institute (Hinston Hall, Cambridge, GB), NBRF (Georgetown University, Medical Centre, Washington DC, USA) and Vecbase (University of Wisconsin, Biotechnology Centre, Madison, Wisconsin, USA).

The process of the present invention can e.g. be used to improve the thermostability of the enzyme phytase.

Once complete DNA sequences of the present invention have been obtained they can be integrated into vectors by methods known in the art and described e.g. in Sambrook et al. (s.a.) to overexpress the encoded polypeptide in appropriate host systems. However, a man skilled in the art knows that also the DNA sequences themselves can be used to transform the suitable host systems of the invention to get overexpression of the encoded polypeptide. Appropriate host systems are for example fungi,

like Aspergilli, e.g. Aspergillus niger [ATCC 9142] or Aspergillus ficuum [NRRL 3135] or like Trichoderma, e.g. Trichoderma reesei; or yeasts, like Saccharomyces, e.g. Saccharomyces cerevisiae or Pichia, like Pichia pastoris, or

5 Hansenula polymorpha, e.g. H. polymorpha (DSM5215); or plants, as described, e.g. by Pen et al., Bio/Technology 11, 811-814 (1994). A man skilled in the art knows that such microorganisms are available from depository authorities, e.g. the American Type Culture Collection (ATCC), the Centraalbureau voor

10 Schimmelcultures (CBS) or the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSM) or any other depository authority as listed in the Journal "Industrial Property" [(1991) 1, pages 29-40]. Bacteria which can be used are e.g. E. coli; Bacilli as, e.g., Bacillus subtilis; or

15 Streptomyces, e.g. Streptomyces lividans (see e.g. Anné and Mallaert in FEMS Microbiol. Lett. 114, 121 (1993). Preferred E. coli strains, which can be used are E. coli K12 strains e.g. M15 [described as DZ 291 by Villarejo et al. in J. Bacteriol. 120, 466-474 (1974)], HB 101 [ATCC No. 33694] or E. coli SG13009

20 [Gottesman et al., J. Bacteriol. 148, 265-273 (1981)].

Vectors which can be used for expression in fungi are known in the art and described e.g. in EP 420358, or by Cullen et al. [Bio/Technology 5, 369-376 (1987)], Ward [Molecular Industrial Mycology, Systems and Applications for Filamentous

25 Fungi, Marcel Dekker, New York (1991)], Upshall et al. [Bio/Technology 5, 1301-1304 (1987)], Gwynne et al. [Bio/Technology 5, 71-79 (1987)], or Punt et al. [J. Biotechnol. 17, 19-34 (1991)]; and for yeasts by Sreekrishna et al. [J. Basic Microbiol. 28, 265-278 (1988), Biochemistry 28, 4117-4125

30 (1989)], Hitzemann et al. [Nature 293, 717-722 (1981)] or in EP 183070, EP 183071, EP 248227, or EP 263311. Suitable vectors

which can be used for expression in E. coli are mentioned, e.g. by Sambrook et al. [s.a.], Fiers et al. [Procd. 8th Int. Biotechnology Symposium", Soc. Franc. de Microbiol., Paris (Durand et al., eds.), pp. 680-697 (1988)], Bujard et al. [Meth. Enzymol. 155, 416-433 (1987)], or Stüber et al. [Immunological Methods, eds. Lefkovits and Pernis, Academic Press, Inc., Vol. IV, 121-152 (1990)]. Vectors that can be used for expression in Bacilli are known in the art and described, e.g. in EP 207459, EP 405370, Proc. Natl. Acad. Sci. USA 81, 439 (1984) or Yansura and Henner, Meth. Enzymol. 185, 199-228 (1990). Vectors which can be used for the expression in H. Polymorpha are known in the art and described, e.g. in Gellissen et al., Biotechnology 9, 291-295 (1991).

Either such vectors already carry regulatory elements, e.g. promotors, or the DNA sequences of the present invention can be engineered to contain such elements. Suitable promotor elements which can be used are known in the art and are, e.g. for Trichoderma reesei the cbh1- [Haarki et al., Biotechnology 7, 596-600 (1989)] or the pki1-promotor [Schindler et al., Gene 130, 271-275 (1993)]; for Aspergillus oryzae the amy-promotor [Christensen et al., Abstr. 19th Lunteren Lectures on Molecular Genetics F23 (1987), Christensen et al., Biotechnology 6, 1419-1422 (1988), Tada et al., Mol. Gen. Genet. 229, 301 (1991)]; and for Aspergillus niger the glaA- [Cullen et al., Bio/Technology 5, 369-376 (1987), Gwynne et al., Bio/Technology 5, 713-719 (1987), Ward in Molecular Industrial Mycology, Systems and Applications for Filamentous Fungi, Marcel Dekker, New York, 83-106 (1991)], alcA- [Gwynne et al., Bio/Technology 5, 718-719 (1987)], suc1- [Boddy et al., Curr. Genet. 24, 60-66 (1993)], aphA- [MacRae et al., Gene 71, 339-348 (1988), MacRae et al., Gene 132, 193-198 (1993)], tpiA- [McKnight et al., Cell 46, 143-

147 (1986), Upshall et al., Bio/Technology 5, 1301-1304 (1987)], gpdA- [Punt et al., Gene 69, 49-57 (1988), Punt et al., J. Biotechnol. 17, 19-37 (1991)] and the pkiA-promotor [de Graaff et al., Curr. Genet. 22, 21-27 (1992)]. Suitable promotor

5 elements that can be used for expression in yeast are known in the art and are, e.g. the pho5-promotor [Vogel et al., Mol. Cell. Biol., 2050-2057 (1989); Rudolf and Hinnen, Proc. Natl. Acad. Sci. 84, 1340-1344 (1987)] or the gap-promotor for expression in Saccharomyces cerevisiae; the aox1-promotor [Koutz

10 et al., Yeast 5, 167-177 (1989); Sreekrishna et al., J. Basic Microbiol. 28, 265-278 (1988)] for Pichia pastoris; or the FMD promoter [Hollenberg et al., EPA No. 0299108] or MOX-promotor [Ledeboer et al., Nucl. Acids Res. 13, 3063-3082 (1985)] for H. polymorpha.

15 Accordingly vectors comprising DNA sequences of the present invention, preferably for the expression of said DNA sequences in bacteria or a fungal or a yeast host and such transformed bacteria or fungal or yeast hosts are also a part of the invention.

20 The invention also provides a system that allows for high expression of proteins, in particular of the phytases of the invention, such as recombinant Hansenula strains. To achieve that, the codons of the DNA sequence of such a protein may be selected on the basis of a codon frequency table of the organism

25 used for expression, e.g. of yeast as in the present case (see e.g. in Example 1). Optionally, the codons for the signal sequence may be selected in a manner as described for the specific case in Example 1; that means that a codon frequency table is prepared on the basis of the codons used in the DNA

30 sequences which encode the amino acid sequences of the given protein family. Then the codons for the design of the DNA

sequence of the signal sequence are selected from a codon frequency table of the host cell used for expression whereby always codons of comparable frequency in both tables are used.

Once such DNA sequences have been expressed in an appropriate host cell in a suitable medium, the encoded protein can be isolated either from the medium in the case the protein is secreted into the medium or from the host organism in case such protein is present intracellularly by methods known in the art of protein purification or described in case of a phytase, e.g. in EP 420358. Accordingly, a process for the preparation of a polypeptide of the present invention wherein transformed bacteria or a host cell as described above are cultured under suitable culture conditions, and the polypeptide is recovered therefrom and a polypeptide when produced by such a process; or a polypeptide encoded by a DNA sequence of the present invention, are also a part of the present invention.

Once obtained, the polypeptides of the present invention can be characterized regarding their properties that make them useful in agriculture by any assay known in the art.

In general, the polypeptides of the present invention can be used without being limited to a specific field of application, e.g. in case of phytases for the conversion of inositol polyphosphates, like phytate, to inositol and inorganic phosphate.

Furthermore, the polypeptides of the present invention can be used in a process for the preparation of a pharmaceutical composition or compound food or feeds wherein the components of such a composition are mixed with at least one polypeptide of the present invention. Accordingly, compound food or feeds or pharmaceutical compositions comprising at least one polypeptide of the present invention are also a part of the present

invention. A man skilled in the art is familiar with their process of preparation. Such pharmaceutical compositions or compound foods or feeds can further comprise additives or components generally used for such purpose and known in the

5 state of the art.

The present invention also provides a process for the reduction of levels of phytate in animal manure wherein an animal is fed such a feed composition in an amount effective in converting phytate contained in the feedstuff to lower inositol

10 phosphates and/or inositol, and inorganic phosphate.

In the present context, a phytase is an enzyme or polypeptide that has phytase activity. A phytase can be e.g. a myo-inositol hexakisphosphate phosphohydrolase, such as (myo-inositol hexakisphosphate 3-phosphohydrolase, EC 3.1.3.8) and

15 (myo-inositol hexakisphosphate 6-phosphohydrolase, EC 3.1.3.26).

In one embodiment, the phytase is purified, viz. at least 85%, preferably at least 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% pure, as evaluated by SDS-PAGE. The phytase may be isolated. Phytase activity can be determined using any

20 phytase assay known in the art, e.g. the assay described herein (see Example 9). The assay temperature may be the optimum temperature of the actual phytase, and the assay pH may be the optimum pH of the actual phytase.

The assay temperature may e.g. be selected within the

25 range of 20-90°C, or 30-80°C, or 35-75°C, for instance temperatures of 37°C, 50°C, 60°C, or 70°C.

The assay pH may e.g. be selected within the range of pH 2-9, or 3-8, or 3-6, for instance assay pH values of 3, 4, 5, 6, or 7 may be chosen.

30 Amino acid sequence homology (or polypeptide or amino acid homology) is determined as the degree of identity between two

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sequences. This may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package [Program Manual for the Wisconsin Package, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin
5 53711, USA], see also Needleman, S.B. and Wunsch, C.D., (1970), J. Mol. Biol., 48, 443-453]. In release 9.1, for comparing polypeptide sequences, the Length Weight is set to 0, and the Gap Weight is set to 3.0.

The degree of identity or homology between two DNA
10 (nucleic acid) sequences may be determined by means of computer programs known in the art such as GAP provided in the GCG program package [Program Manual for the Wisconsin Package, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin 53711, USA), see also Needleman, S.B. and Wunsch, C.D., (1970),
15 J. Mol. Biol., 48, 443-453]. In release 9.1, GAP is used with the following settings for DNA sequence comparison: GAP creation penalty of 50 and GAP extension penalty of 3.

Suitable experimental conditions for determining whether a given DNA or RNA sequence hybridizes to a specified nucleotide
20 or oligonucleotide probe involves presoaking of the filter containing the DNA or RNA fragments to examine for hybridization in 5 x SSC (Sodium chloride/Sodium citrate; (J. Sambrook, E.F. Fritsch, and T. Maniatis, 1989, Molecular Cloning, A Laboratory Manual, 2nd edition, Cold Spring Harbor, New York) for 10 min,
25 and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution, 0.5 % SDS and 100 μg/ml of denatured sonicated salmon sperm DNA (Sambrook et al. 1989), followed by hybridization in the same solution containing a concentration of 10 ng/ml of a random-primed (Feinberg, A. P. and Vogelstein, B.
30 (1983) Anal. Biochem. 132:6-13), ^{32}P-dCTP-labeled (specific

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activity > 1 x 10^9 cpm/μg) probe for 12 hours at approximately 45°C.

The filter is then washed twice for 30 minutes in 2 x SSC, 0.5 % SDS at at least 55°C (low stringency), at at least 60°C
5 (medium stringency), at at least 65°C (medium/high stringency), at at least 70°C (high stringency), or at at least 75°C (very high stringency).

Molecules to which the oligonucleotide probe hybridizes under these conditions can be detected using an x-ray film.

10 Phytases of amended thermostability, or thermostable phytases, are one aspect of the present invention. A ''thermostable'' phytase is a phytase that has a Tm (melting temperature) - as measured on purified phytase protein by Differential Scanning Calorimetry (DSC) - of at least 65°C. For
15 the DSC, a constant heating rate may be used, e.g. of 10°C/min. In alternative embodiments, the Tm is at least 66, 67, 68, 69, 70, 71, 72, 73, 74 or 75°C. Or, the Tm is equal to or lower than 150°C, or equal to or lower than 145, 140, 135, 130, 125, 120, 115 or 110°C. Accordingly, examples of intervals of Tm are: 65-
20 150°C, 66-150°C, - (etc.) - 75-150°C; 65-145°C, 66-145°C, - (etc.) - 75-145°C; 65-140°C, - (etc.) - 75-140°C; - (etc.) - 65-110°C, 66-110°C, - (etc.) - 75-110°C.

Particular ranges for Tm are the following: between 65 and 110°C; between 70 and 110°C; between 70 and 100°C; between 75
25 and 95°C, or between 80 and 90°C.

In Examples 9 and 10 below, the measurement of Tm by DSC is described, and the Tm's of a number of phytases are shown.

The optimum temperatures are also indicated, since - as an alternative mean - a thermostable phytase can be defined as a
30 phytase having a temperature-optimum of at least 60°C. Preferably, the optimum temperature is determined on the

substrate phytate or phytic acid at pH 5.0 or 5.5. Example 9 describes an example of a phytase assay, including a definition of units.

In alternative embodiments, the optimum temperature is at
5 least 61, 62, 63, 64, 65, 66, 67, 68, 69 or 70°C. In a particular embodiment, the optimum temperature is equal to or lower than 140°C, or equal to or lower than 135, 130, 125, 120, 115, 110, 105 or 100°C. Accordingly, examples of intervals of optimum temperature are: 60-140°C, 61-140°C, - (etc.) - 70-
10 140°C; 60-135°C, 61-135°C, - (etc.) - 70-135°C; 60-130°C, - (etc.) - 70-130°C; - (etc.) - 60-100°C, 61-100°C, - (etc.) - 70-100°C.

Before describing the present invention in more detail a short explanation of the Figures enclosed is given below.

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Figure 1: Design of the consensus phytase-1 sequence. The letters represent the amino acid residues in the one-letter code. The following sequences were used for the alignment: phyA from Aspergillus terreus 9A-1 [Mitchell, D. B., Vogel, K.,
20 Weimann, B. J., Pasamontes, L. & van Loon, A. P. G. M. (1997) The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi Aspergillus terreus and Myceliophthora thermophila, Microbiology 143, 245-252); from amino acid (aa) 27; SEQ ID NO: 1]; phyA from A.
25 terreus cbs116.46 [EP 897985]. A heat resistant phytase of Aspergillus fumigatus with superior performance in animal experiments. Phytase optimization and natural variability. In: The Biochemistry of phytate and phytases (eds. Rasmussen, S.K; Raboy, V.; Dalbøge, H. and Loewus, F.; Kluwer Academic
30 Publishers); from aa 27; SEQ ID NO: 2; phyA from Aspergillus niger var. awamori (Piddington et al (1993) Gene 133, 55-62;

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from aa 27; SEQ ID NO: 3); phyA from A. niger T213 (EP 897985); from aa 27; SEQ ID NO: 4); phyA from A. niger strain NRRL3135 [van Hartingsveldt, W., van Zeijl, C. M. F., Harteveld, G. M., Gouka, R. J., Suykerbuyk, M. E. G., Luiten, R. G. M., van

5 Paridon, P. A., Selten, G. C. M., Veenstra, A. E., van Gorcom, R. F. M., & van den Hondel, C. A. M. J. J. (1993) Cloning, characterization and overexpression of the phytase-encoding gene (phyA) of Aspergillus niger. Gene 127, 87-94; from aa 27; SEQ ID NO: 5]; phyA from Aspergillus fumigatus ATCC 13073 (Pasamontes,

10 L., Haiker, M., Wyss, M., Tessier, M. & van Loon, A. P. G. M. (1997) Cloning, purification and characterization of a heat stable phytase from the fungus Aspergillus fumigatus, Appl. Environ. Microbiol. 63, 1696-1700; from aa 25; SEQ ID NO: 6]; phyA from A. fumigatus ATCC 32722 (EP 897985); from aa 27; SEQ

15 ID NO: 7); phyA from A. fumigatus ATCC 58128 (EP 897985); from aa 27; SEQ ID NO: 8); phyA from A. fumigatus ATCC 26906 (EP 897985); from aa 27; SEQ ID NO: 9); phyA from A. fumigatus ATCC 32239 (EP 897985); from aa 30; SEQ ID NO: 10; phyA from Emericella nidulans [Pasamontes, L., Haiker, M., Henriquez-

20 Huecas, M., Mitchell, D. B. & van Loon, A. P. G. M. (1997a). Cloning of the phytases from Emericella nidulans and the thermophilic fungus Talaromyces thermophilus. Biochim. Biophys. Acta 1353, 217-223; from aa 25; SEQ ID NO: 11]; phyA from Talaromyces thermophilus (Pasamontes et al., 1997a; from aa 24;

25 SEQ ID NO: 12); and phyA from Myceliophthora thermophila (Mitchell et al., 1997; from aa 19; SEQ ID NO: 13). The alignment was calculated using the program PILEUP. The location of the gaps was refined by hand. Capitalized amino acid residues in the alignment at a given position belong to the amino acid

30 coalition that establish the consensus residue. In bold, beneath the calculated consensus sequence, the amino acid sequence of

the finally constructed consensus phytase (Fcp) is shown (SEQ ID NO: 14). The gaps in the calculated consensus sequence were filled by hand according to principals stated in Example 1.

5   Figure 2: DNA sequence (SEQ ID NO: 15) of the consensus phytase-1 gene (fcp) and of the primers used for the gene construction. The calculated amino acid sequence (Figure 1, SEQ ID NO: 14) was converted into a DNA sequence using the program BACKTRANSLATE [Devereux, J., Haeberli, P. & Smithies, O. (1984) 10 A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. 12, 387-395], and the codon frequency table of highly expressed yeast genes (GCG program package, 9.0). The signal peptide of the phytase from A. terreus cbs 116.46 was fused to the N-terminus. The amino acid sequence shown in Fig. 2 15 is SEQ ID NO: 16. The bold bases represent the sequences of the oligonucleotides used to generate the gene. The names of the respective oligonucleotides are alternately noted above or below the sequence. The underlined bases represent the start and stop codon of the gene. The bases written in italics represent the 20 two introduced Eco RI sites.

Figure 3: Alignment and consensus sequence of five Basidiomycete phytases. The letters represent the amino acid residues in the one-letter code. The amino acid sequences of the 25 phytases from Paxillus involutus, phyA1 (from aa 21; SEQ ID NO: 17; and phyA2 (from aa 21, WO 98/28409; SEQ ID NO: 18); Trametes pubescens (from aa 24, WO 98/28409; SEQ ID NO: 19); Agrocybe pediades (from aa 19, WO 98/28409; SEQ ID NO: 20); and Peniophora lycii (from aa 21, WO 98/28409; SEQ ID NO: 21), 30 starting with the amino acid residues mentioned in parentheses, were used for the alignment and the calculation of the

corresponding consensus sequence called "Basidio" (Example 2; SEQ ID NO: 22). The alignment was performed with the program PILEPUP. The location of the gaps was refined by hand. The consensus sequence was calculated by the program PRETTY. While a vote weight of 0.5 was assigned to the two P. involutus phytases, all other genes were used with a vote weight of 1.0 for the consensus sequence calculation. At positions where the program was not able to determine a consensus residue, the Basidio sequence contains a dash. Capitalized amino acid residues in the alignment at a given position represent the amino acid coalition that established the consensus residue.

Figure 4: Design of consensus phytase-10 amino acid sequence. By adding the sequence of Thermomyces lanuginosus phytase [Berka, R. M., Rey, M. W., Brown, K. M., Byun, T. & Klotz, A. V. (1998) Molecular characterization and expression of a phytase gene from the thermophilic fungus Thermomyces lanuginosus. Appl. Environ. Microbiol. 64, 4423-4427; SEQ ID NO: 23] and the consensus sequence of the phytases from five Basidiomycetes (SEQ ID NO: 22) to the alignment of Figure 1, an improved consensus sequence was calculated by the program PRETTY. Additionally, the amino acid sequence of A. niger T213 was omitted, and a vote weight of 0.5 was assigned to the remaining two A. niger phytase sequences. For further information see Example 2.

Figure 5: DNA and amino acid sequence of consensus phytase-10 (SEQ ID NO: 25, and SEQ ID NO: 26, respectively). The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The sequence of the oligonucleotides that were used to assemble the gene are in

bold letters. The names of the respective oligonucleotides and the amino acids that differ relative to consensus phytase-1 are underlined. The fcp10 gene was assembled from the following oligonucleotides: CP-1, CP-2, CP-3.10, CP-4.10, CP-5.10, CP-6,

5 CP-7.10, CP-8.10, CP-9.10, CP-10.10, CP-11.10, CP-12.10, CP-13.10, CP-14.10, CP-15.10, CP-16.10, CP-17.10, CP18.10, CP-19.10, CP-20.10, CP-21.10, and CP-22.10. The newly synthesized oligonucleotides are additionally marked by the number 10. The phytase contains the following 32 exchanges relative to

10 consensus phytase-1: Y54F, E58A, D69K, D70G, A94K, N134Q, I158V, S187A, Q188N, D197N, S204A, T214L, D220E, L234V, A238P, D246H, T251N, Y259N, E267D, E277Q, A283D, R291I, A320V, R329H, S364T, I366V, A379K, S396A, G404A, Q415E, A437G, A463E. The underlined mutations revealed a stabilizing effect on consensus phytase-1

15 when tested as single mutations in consensus phytase-1.

Figure 6: Alignment for the design of consensus phytase-11 (SEQ ID NO: 27). In contrast to the design of consensus phytase-10, for the design of the amino acid sequence of consensus

20 phytase-11, all Basidiomycete phytases were used as independent sequences using an assigned vote weight of 0.2 for each Basidiomycete sequence. Additionally, the amino acid sequence of A. niger T213 was again used in this alignment.

25 Figure 7: DNA and amino acid sequence of consensus phytase-1-thermo[8]-Q50T-K91A (SEQ ID NO: 28, and SEQ ID NO: 29, respectively). The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The replaced amino acid residues (relative to consensus phytase-1)

30 are underlined. The stop codon of the gene is marked by a star (*).

Figure 8: DNA and amino acid sequence of consensus phytase-10-thermo[3]-Q50T-K91A (SEQ ID NO: 30, and SEQ ID NO: 31, respectively). The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The replaced amino acid residues (relative to consensus phytase-10) are underlined. The stop codon of the gene is marked by a star (*).

Figure 9: DNA and amino acid sequence of A. fumigatus ATCC 13073 phytase alpha-mutant Q51T (SEQ ID NO: 32, and SEQ ID NO: 33, respectively). The amino acid sequence is written above the corresponding DNA sequence using the one-letter code. The replaced amino acid residues (relative to A. fumigatus ATCC 13073 phytase) are underlined. The stop codon of the gene is marked by a star (*).

Figure 10: DNA and amino acid sequence of consensus phytase-7 (SEQ ID NO: 34, and SEQ ID NO: 35, respectively). The amino acids are written above the corresponding DNA sequence using the one-letter code. The sequence of the oligonucleotides used to assemble the gene are in bold letters. Oligonucleotides and amino acids that were exchanged (relative to consensus phytase-1) are underlined and the corresponding triplets are written in small case letters. The fcp7 gene was assembled from the following oligonucleotides: CP-1, CP-2, CP-3, CP-4.7, CP-5.7, CP-6, CP-7, CP-8.7, CP-9, CP-10.7, CP-11.7, CP-12.7, CP-13.7, CP-14.7, CP-15.7, CP-16, CP-17.7, CP-18.7, CP-19.7, CP-20, CP-21, and CP-22. The newly synthesized oligonucleotides are additionally marked by the number 7. Consensus phytase-7 contains the following 24 exchanges in comparison to the

original consensus phytase-1: S89D, S92G, A94K, D164S, P201S, G203A, G205S, H212P, G224A, D226T, E255T, D256E, V258T, P265S, Q292H, G300K, Y305H, A314T, S364G, M365I, A397S, S398A, G404A, and A405S.

5

Figure 11: Differential scanning calorimetry (DSC) of consensus phytase-1 and consensus phytase-10. The protein samples were concentrated to about 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant
10 heating rate of 10°C/min was applied up to 95°C. DSC of consensus phytase-10 (upper graph) yielded a melting temperature of 85.4°C, which is 7.3°C higher than the melting point of consensus phytase-1 (78.1°C, lower graph).

15 Figure 12: Differential scanning calorimetry (DSC) of consensus phytase-10-thermo[3]-Q50T and consensus phytase-10-thermo[3]-Q50T-K91A. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10°C/min was applied
20 up to 95°C. DSC of consensus phytase-10-thermo[3]-Q50T (upper graph) yielded a melting temperature of 88.6°C, while the melting temperature of consensus phytase-10-thermo-Q50T-K91A was determined to be 89.3°C.

25 Figure 13: Comparison of the temperature optimum between consensus phytase-1, consensus phytase-10 and consensus phytase-10-thermo[3]-Q50T. For the determination of the temperature optimum, the phytase standard assay of Example 9 was performed at a series of temperatures between 37 and 86°C. The diluted
30 supernatant of transformed S. cerevisiae strains was used for the determination. The other components of the supernatant had

no influence on the determination of the temperature optimum: ∧, consensus phytase-1; ◇, consensus phytase-10; ■, consensus phytase 10-thermo[3]-Q50T.

5   Figure 14: pH-dependent activity profile and substrate specificity of consensus phytase-10 and its variants thermo[3]-Q50T and thermo[3]-Q50T-K91A. The phytase activity was determined using the standard assay in appropriate buffers (see Example 9) at different pH-values. Graph a) shows the pH-

10 dependent activity profile of consensus phytase-10 (□), consensus phytase-10-thermo[3]-Q50T (•), and consensus phytase-10-thermo[3]-Q50T-K91A (∧). Graph b) shows the corresponding substrate specificity tested by replacement of phytate in the standard assay by the indicated compounds; open bars, consensus

15 phytase-10; grey bars, consensus phytase-10-thermo[3]-Q50T; dark bars, consensus phytase-10-thermo[3]-Q50T-K91A). The numbers correspond to the following substrates: 1, phytate; 2, p-nitrophenyl phosphate; 3, phenyl phosphate; 4, fructose-1,6-bisphosphate; 5, fructose-6-phosphate; 6, glucose-6-phosphate;

20 7, ribose-5-phosphate; 8, DL-glycerol-3-phosphate; 9, glycerol-2-phosphate; 10, 3-phosphoglycerate; 11, phosphoenolpyruvate; 12, AMP; 13, ADP; 14, ATP.

    Figure 15: pH-dependent activity profile and substrate

25 specificity of consensus phytase-1-thermo[8]-Q50T and of consensus phytase-1-thermo[8]-Q50T-K91A. The phytase activity was determined using the standard assay in appropriate buffers (see Example 9) at different pH-values. Graph a) shows the pH-dependent activity profile of the Q50T- (■) and the Q50T-K91A-

variant (.). Graph b) shows the corresponding substrate specificities tested by replacement of phytate in the standard assay by the indicated compounds (open bars, consensus phytase-1-thermo[8]-Q50T; filled bars, consensus phytase-1-thermo[8]-

5 Q50T-K91A). The substrates are listed in the legend of Figure 14.

Figure 16: Differential scanning calorimetry (DSC) of consensus phytase-1-thermo[8]-Q50T and consensus phytase-1-

10 thermo[8]-Q50T-K91A. The protein samples were concentrated to ca. 50-60 mg/ml and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10°C/min was applied up to 95°C. DSC of consensus phytase-1-thermo[8]-Q50T (upper graph) showed a melting temperature of 84.7°C, while the melting

15 point of consensus phytase-1-thermo[8]-Q50T-K91A was found at 85.7°C.

Figure 17: Comparison of the temperature optimum between consensus phytase-1, consensus phytase-1-thermo[3] and consensus

20 phytase-1-thermo[8]. For the determination of the temperature optimum, the phytase standard assay was performed at a series of temperatures between 37 and 86°C. Protein purified from the supernatant of transformed S. cerevisiae strains was used for the determination. O, consensus phytase-1; □, consensus

25 phytase-1-thermo[3]; ▲, consensus phytase 1-thermo[8].

Figure 18: Comparison of the pH-dependent activity profile and substrate specificity between consensus phytase-1, consensus phytase-7, and the phytase from A. niger NRRL 3135. The phytase

30 activity was determined using the standard assay in appropriate buffers (see Example 9) at different pH-values. Graph a) shows

the pH-dependent activity profile of consensus phytase-1 (■), the phytase from A. niger NRRL 3135 (O), and of consensus phytase-7 (▲). Graph b) shows the corresponding substrate specificities tested by replacement of phytate in the standard
5 assay by the indicated compounds (black bars, A. niger NRRL 3135 phytase; open bars, consensus phytase-1; dashed bars, consensus phytase-7). The substrates are listed in the legend of Figure 14.

10   Figure 19: Differential scanning calorimetry (DSC) of the phytase from A. fumigatus ATCC 13073 and of its stabilized alpha-mutant, which contains the following amino acid exchanges: F55Y, V100I, F114Y, A243L, S265P, and N294D.

    The protein samples were concentrated to ca. 50-60 mg/ml
15 and extensively dialyzed against 10 mM sodium acetate, pH 5.0. A constant heating rate of 10°C/min was applied up to 95°C. DSC of A. fumigatus 13073 phytase (lower graph) revealed a melting temperature of 62.5°C, while the melting point of the alpha-mutant was found at 67.0°C.

20

    Figure 20: Comparison of the temperature optima of A. fumigatus 13073 wild-type phytase, its alpha-mutant, and a further stabilized alpha-mutant (E59A-S154N-R329H-S364T-G404A). For the determination of the temperature optimum, the phytase
25 standard assay was performed at a series of temperatures between 37 and 75°C. The diluted supernatant of transformed S. cerevisiae strains was used for the determination. The other components of the supernatant had no influence on the determination of the temperature optimum. O, A. fumigatus ATCC
30 13073 phytase; ▲, A. fumigatus ATCC 13073 alpha-mutant; □, A.

fumigatus ATCC 13073 alpha-mutant-(E59A-S154N-R329H-S364T-G404A)-Q27T; ■, A. fumigatus ATCC 13073 alpha-mutant-(E59A-S154N-R329H-S364T-G404A)-Q51T-K92A. Q51T and K92A correspond to consensus phytase-1 substitutions Q50T and K91A, respectively.

Figure 21: Amino acid sequence of consensus phytase-12 (consphy12; SEQ ID NO: 36) which contains a number of active site residues transferred from the "basidio" consensus sequence to consensus phytase-10-thermo[3]-Q50T-K91A (underlined).

Figure 22: DNA and amino acid sequence of consensus phytase-3-thermo[11]-Q50T. The amino acids are written below the corresponding DNA sequence using the one-letter code.

Figure 23: DNA and amino acid sequence of consensus phytase-3-thermo[11]-Q50T-K91A. The amino acids are written below the corresponding DNA sequence using the one-letter code.

Figure 24: DNA and amino acid sequence of consensus phytase-10-thermo[5]-Q50T. The amino acids are written below the corresponding DNA sequence using the one-letter code.

Figure 25: DNA and amino acid sequence of consensus phytase-10-thermo[5]-Q50T-K91A. The amino acids are written below the corresponding DNA sequence using the one-letter code.

The phytase-producing microorganism strains mentioned herein, viz. Paxillus involutus CBS 100231; Peniophora lycii CBS 686.96; Agrocybe pediades CBS 900.96; and Trametes pubescens CBS 100232; were isolated from natural samples originating from, respectively, Denmark; Denmark; Denmark; and Sweden (the Uppsala

collection. The samples were collected in November 1992; October 1993; June 1995; and in November 1995, respectively.

Example 1

Consensus phytase-1

The amino acid sequence of consensus phytase-1 (fungal consensus phytase, fcp) was designed and calculated as described in Examples 1 and 2 of EP 897985. Table 1 below shows the origin and vote weight of the phytase amino acid sequences used for the design of consensus phytase-1. The consensus phytase-1 sequence was furthermore converted into a DNA sequence as described in Example 3 of EP 897985, and the consensus phytase-1 gene was constructed and cloned as described in Example 4 of EP 897985.

Table 1

Origin and vote weight of the phytase amino acid sequences

- phyA from Aspergillus terreus 9A-1, aa 27, vote weight 0.5 (Mitchell et al., 1997)

- phyA from Aspergillus terreus cbs116.46, aa 27, vote weight 0.5 (EP 897985)

- phyA from Aspergillus niger var. awamori, aa 27, vote weight 0.33 [Piddington, C. S., Houston, C. S., Paloheimo, M., Cantrell, M., Miettinen-Oinonen, A., Nevalainen, H., & Rambosek, J. (1993) The cloning and sequencing of the genes encoding phytase (phy) and pH 2.5-optimum acid phosphatase (aph) from Aspergillus niger var. awamori. Gene 133, 55-62].

- phyA from Aspergillus niger T213 (EP 897985), aa 27, vote weight 0.33

- phyA from Aspergillus niger strain NRRL3135, aa 27, vote weight 0.33 (van Hartingsveldt et al., 1993)

- phyA from Aspergillus fumigatus ATCC 13073, aa 26, vote weight 0.2 (Pasamontes et al., 1997)

- phyA from Aspergillus fumigatus ATCC 32722, aa 26, vote weight 0.2 (EP 897985)

5 - phyA from Aspergillus fumigatus ATCC 58128, aa 26, vote weight 0.2 (EP 897985)

- phyA from Aspergillus fumigatus ATCC 26906, aa 26, vote weight 0.2 (EP 897985)

- phyA from Aspergillus fumigatus ATCC 32239, aa 30, vote weight

10 0.2 (EP 897985)

- phyA from Emericella nidulans , aa 25, vote weight 1.0 (Pasamontes et al., 1997a)

- phyA from Talaromyces thermophilus ATCC 20186, aa 24, vote weight 1.0 (Pasamontes et al., 1997a)

15 - phyA from Myceliophthora thermophila, aa 19, vote weight 1.0 (Mitchell et al., 1997)

Example 2

Design of an improved consensus phytase (consensus

20 phytase-10) amino acid sequence

The alignments used for the design of consensus phytase-10 were calculated using the program PILEUP from the GCG Sequence Analysis Package Release 9.0 (Devereux et al., 1984) with the

25 standard parameters (gap creation penalty 12, gap extension penalty 4). The location of the gaps was refined using a text editor.

The following sequences were used for the alignment of the Basiodiomycete phytases starting with the amino acid (aa)

30 mentioned in Table 2:

Table 2

Origin and vote weight of five Basidiomycete phytases used for the calculation of the corresponding consensus amino acid sequence (basidio)

5

- phyA1 from Paxillus involutus CBS No. 100231, aa 21, vote weight 0.5 (WO 98/28409)

- phyA2 from Paxillus involutus CBS No. 100231, aa 21, vote weight 0.5 (WO 98/28409)

10 - phyA from Trametes pubescens CBS No. 100232, aa 24, vote weight 1.0 (WO 98/28409)

- phyA from Agrocybe pediades CBS No. 900.96, aa 19, vote weight 1.0 (WO 98/28409)

- phyA from Peniophora lycii CBS No. 686.96, aa 21, vote weight 15 1.0 (WO 98/28409)

The alignment is shown in Figure 3.

In Table 3 the genes that were used for the final 20 alignment are listed. The first amino acid (aa) of the sequence that is used in the alignment is mentioned behind the organism's designation.

Table 3

25 Origin and vote weight of the phytase sequences used for the design of consensus phytase-10

- phyA from Aspergillus terreus 9A-1, aa 27, vote weight 0.5 (Mitchell et al., 1997)

30 - phyA from Aspergillus terreus cbs116.46, aa 27, vote weight 0.5 (EP 897985)

- phyA from Aspergillus niger var. awamori, aa 27, vote weight 0.5 (Piddington et al., 1993)

- phyA from Aspergillus niger strain NRRL3135, aa 27, vote weight 0.5 (van Hartingsveldt et al., 1993)

5 - phyA from Aspergillus fumigatus ATCC 13073, aa 26, vote weight 0.2 (Pasamontes et al., 1997)

- phyA from Aspergillus fumigatus ATCC 32722, aa 26, vote weight 0.2 (EP 897985)

- phyA from Aspergillus fumigatus ATCC 58128, aa 26, vote weight 10 0.2 (EP 897985)

- phyA from Aspergillus fumigatus ATCC 26906, aa 26, vote weight 0.2 (EP 897985)

- phyA from Aspergillus fumigatus ATCC 32239, aa 30, vote weight 0.2 (EP 897985)

15 - phyA from Emericella nidulans , aa 25, vote weight 1.0 (Pasamontes et al., 1997a)

- phyA from Talaromyces thermophilus ATCC 20186, aa 24, vote weight 1.0 (Pasamontes et al., 1997a)

- phyA from Myceliophthora thermophila, aa 19, vote weight 1.0 20 (Mitchell et al., 1997)

- phyA from Thermomyces lanuginosus, aa 36, vote weight 1.0 (Berka et al., 1998)

- Consensus sequence of five Basidiomycete phytases, vote weight 1.0 (Basidio, Figure 3)

25

The corresponding alignment is shown in Figure 4.

Calculation of the amino acid sequence of consensus phytase-10

30   To improve the alignment, we added the original consensus sequence of five phytases from four different Basidiomycetes

(called Basidio; still containing the undefined sequence positions; see Figure 3), nearly all phytase sequences used for the calculation of the original consensus phytase sequences and one new phytase sequence from the Ascomycete Thermomyces

5 lanuginosus to a larger alignment.

We set plurality on 2.0 and threshold on 3. The used vote weights are listed in Table 3. The alignment and the corresponding consensus sequence are presented in Figure 4. The new consensus phytase sequence has 32 different amino acids in

10 comparison to the original consensus phytase-1. Positions for which the program PRETTY was not able to calculate a consensus amino acid residue were filled according to rules mentioned in Example 1. None of the residues suggested by the program was replaced.

15 Furthermore, in another calculation, we included all Basidiomycete phytases as single amino acid sequences but assigning a vote weight of 0.2 in the calculation. The corresponding alignment is shown in Figure 6. The calculated consensus amino acid sequence (consensus phytase-11) has the

20 following differences to the sequence of consensus phytase-10. Letter X means that the program was not able to calculate a consensus amino acid; the amino acid in parenthesis corresponds to the amino acid finally included into consensus phytase-10.

D35X (first letter for consensus phytase-10, last letter

25 for consensus phytase-11), X(K)69K, X(E)100E, A101R, Q134N, X(K)153N, X(H)190H, X(A)204S, X(E)220D, E222T, V227A, X(R)271R, H287A, X(D)288D, X(K)379K, X(I)389I, E390X, X(E)415E, X(A)416A, X(R)446L, E463A. The numbering is as in Fig. 5.

We also checked single amino acid replacements suggested

30 by the improved consensus sequences 10 and 11 on their influence

33

on the stability of the original consensus phytase-1. The approach is described in example 3.

Conversion of the consensus phytase-10 amino acid sequence
5 into a DNA sequence

The first 26 amino acid residues of A. terreus cbs116.46 phytase were used as signal peptide and fused to the N-terminus of consensus phytase-10. The used procedure is further described in Example 1.

10   The resulting sequence of the fcp10 gene is shown in Figure 5.

Construction and cloning of the consensus phytase-10 gene (fcp10)

15   The calculated DNA sequence of fcp10 was divided into oligonucleotides of 85 bp, alternately using the sequence of the sense and the anti-sense strand. Every oligonucleotide overlaps 20 bp with the previous and the following oligonucleotide of the opposite strand. The location of all primers, purchased from 20 Microsynth, Balgach (Switzerland) and obtained in a PAGE-purified form, is indicated in Figure 5.

PCR-Reactions

In three PCR reactions, the synthesized oligonucleotides 25 were composed to the entire gene. For the PCR, the High Fidelity Kit from Boehringer Mannheim (Boehringer Mannheim, Mannheim, Germany) and the thermo cycler ''The ProtokolTM'' from AMS Biotechnology (Europe) Ltd. (Lugano, Switzerland) were used. The following oligonucleotides were used in a concentration of 0.2 30 pMol/ml.

Mix 1.10:   CP-1, CP-2, CP-3.10, CP-4.10, CP-5.10, CP-6,
             CP-7.10, CP-8.10, CP-9.10, CP-10.10

Mix 2.10:   CP-9.10, CP-11.10, CP-12.10, CP-13.10, CP-
             14.10, CP-15.10, CP-16.10, CP-17.10, CP18.10,
             CP-19.10, CP-20.10, CP-21.10, CP-22.10

The newly synthesized oligonucleotides are marked by the number 10. Consensus phytase-10 contains the following 32 exchanges, which are underlined in Figure 5, in comparison to the original consensus phytase-1: Y54F, E58A, D69K, D70G, A94K, N134Q, I158V, S187A, Q188N, D197N, S204A, T214L, D220E, L234V, A238P, D246H, T251N, Y259N, E267D, E277Q, A283D, R291I, A320V, R329H, S364T, I366V, A379K, S396A, G404A, Q415E, A437G, A463E.

Four short PCR primers were used for the assembling of the oligonucleotides:

CP-a: *Eco RI*

    5'-TATAT*GAATTC*ATGGGCGTGTTCGTC-3' (SEQ ID NO: 37)

CP-b:

    5'-TGAAAAGTTCATTGAAGGTTTC-3' (SEQ ID NO: 38)

CP-c.10:

    5'-TCTTCGAAAGCAGTACACAAAC-3' (SEQ ID NO: 39)

CP-e: *Eco RI*

    5'-TATAT*GAATTC*TTAAGCGAAAC-3' (SEQ ID NO: 40)

PCR reaction a: 10 μl Mix 1.10 (2.0 pmol of each oligonucleotide)

2 μl nucleotides (10 mM of each nucleotide)

2 μl primer CP-a (10 pmol/ml)

5   2 μl primer CP-c.10 (10 pmol/ml)

10,0 μl PCR buffer

0.75 μl polymerase mixture (2.6 U)

73.25 μl H_2O

10 PCR reaction b: 10 μl Mix 2.10 (2.0 pmol of each oligonucleotide)

2 μl nucleotides (10 mM each nucleotide)

2 μl primer CP-b (10 pmol/ml)

2 μl primer CP-e (10 pmol/ml)

15   10,0 μl PCR buffer

0.75 μl polymerase mixture (2.6 U)

73.25 μl H_2O

Reaction conditions for PCR reactions a and b:

20   step 1   2 min - 45°C

step 2   30 sec - 72°C

step 3   30 sec - 94°C

step 4   30 sec - 52°C

step 5   1 min - 72°C

25

Steps 3 to 5 were repeated 40-times.

The PCR products (670 and 905 bp) were purified by agarose gel electrophoresis (0.9% agarose), followed by gel extraction 30 (QIAEX II Gel Extraction Kit, Qiagen, Hilden, Germany). The purified DNA fragments were used for the PCR reaction c.

PCR reaction c: 6 μl PCR product of reaction a $\approx$50 ng)

6 μl PCR product of reaction b $\approx$50 ng)

2 μl primer CP-a (10 pmol/ml)

5   2 μl primer CP-e (10 pmol/ml)

10,0 μl PCR buffer

0.75 μl polymerase mixture (2.6 U)

73.25 μl H$_2$O

10 Reaction conditions for PCR reaction c:

        step 1   2 min - 94°C

        step 2   30 sec - 94°C

        step 3   30 sec - 55°C

        step 4   1 min - 72°C

15

Steps 2 to 4 were repeated 31-times.

The resulting PCR product (1.4 kb) was purified as mentioned above, digested with EcoRI, and ligated in an EcoRI-
20 digested and dephosphorylated pBsk(-)-vector (Stratagene, La Jolla, CA, USA). 1 μl of the ligation mixture was used to transform E. coli XL-1 competent cells (Stratagene, La Jolla, CA, USA). All standard procedures were carried out as described by Sambrook et al. (1987). The DNA sequence of the constructed
25 gene (fcp10) was checked by sequencing as known in the art.

Example 3

Increasing the thermostability of consensus phytase-1 by introduction of single mutations suggested by the amino acid sequences of consensus phytase-10 and consensus phytase-11

In order to increase the thermostability of homologous genes, it is also possible to test the stability effect of each differing amino acid residue between the protein of interest and the calculated consensus sequence and to combine all stabilizing mutations into the protein of interest. We used the consensus phytase-1 as protein of interest and tested the effect on the protein stability of 34 amino acid residues that differ relative to consensus phytase-10 and/or -11 by single site-directed mutagenesis.

To construct muteins for expression in A. niger, S. cerevisiae, or H. polymorpha, the corresponding expression plasmid containing the consensus phytase-1 gene was used as template for site-directed mutagenesis (see Examples 6-8). Mutations were introduced using the "quick exchangeTM site-directed mutagenesis kit" from Stratagene (La Jolla, CA, USA) following the manufacturer's protocol and using the corresponding primers. All mutations made and the corresponding primers are summarized in Table 4. Plasmids harboring the desired mutation were identified by DNA sequence analysis as known in the art.

38

Table 4

Primers used for site-directed mutagenesis of consensus phytase-1

Exchanged bases are highlighted in bold. The introduction of a
5 restriction site is marked above the sequence. When a
restriction site is written in parenthesis, the mentioned site
was destroyed by introduction of the mutation.

    mutation   Primer set

10
                             Kpn I
    Q50T   5'-CACTTGTGG*GGTACC*TACTCTCCATACTTCTC-3' (SEQ ID NO: 41)
            5'-GAGAAGTATGGAGAGTA*GGTA*CCCCACAAGTG-3'

    Y54F   5'-GGTCAATACTCTCCATTCT**T**CTCTTTGGAAG-3' (SEQ ID NO: 42)
15   5'-CTTCCAAAGAGAAG**A**ATGGAGAGTATTGACC-3'

    E58A   5'-CATACTTCTCTTTGG**C**AGACGAATCTGC-3' (SEQ ID NO: 43)
            5'-GCAGATTCGTCT**G**CCAAAGAGAAGTATG-3'

20   *Aat* II
    D69K   5'-CTCCA*GACGT*C**CCAAAG**GACTGTAGAGTTAC-3' (SEQ ID NO: 44)
            5'-GTAACTCTACAGTC**CTTT**GG*GACGTC*TGGAG-3'

                       Aat II
25 D70G   5'-CTCCA*GACGT*C**CC**AGACG**G**CTGTAGAGTTAC-3' (SEQ ID NO: 45)
            5'-GTAACTCTACAG**C**CGTCTGG*GACGTC*TGGAG-3'

    K91A   5'-GATACCCAACTTCTTCT**GC**GTCTAAGGCTTACTCTG-3'
                                       (SEQ ID NO: 46)
30   5'-CAGAGTAAGCCTTAGAC**GC**AGAAGAAGTTGGGTATC-3'

                       Sca I
    A94K   5'-CTTCTAAGTCTAAG**AAG**TACT*C*TGCTTTG-3' (SEQ ID NO:47)
            5'-CAAAGCAGA*GTACT*T**CTT**CTTAGACTTAGAAG-3'
35
    A101R   5'-GCTTACTCTGCTTTGATTGAA**CGG**ATTCAAAAGAACGCTAC-3'
                                        (SEQ ID NO: 48)
            5'-GTAGCGTTCTTTTGAAT**CCG**TTCAATCAAAGCAGAGTAAGC-3'

40 N134Q   5'-CCATTCGGTGAA**CAG**CAAATGGTTAACTC-3' (SEQ ID NO: 49)
            5'-GAGTTAACCATTTG**CTG**TTCACCGAATGG-3'

39

Nru I

K153N 5'-GATACAAGGCT**C**TCGCGAGAAA**C**ATTGTTC -3' (SEQ ID NO: 50)
5'-GGAACAAT**G**TTTCTCGCGAGAGCCTTGTATC-3'

5

Bss HI

I158V 5'-GATTGTTCCATTC**G**TGCGCGCTTCTGGTTC-3' (SEQ ID NO: 51)
5'-GAACCAGAAGCGCGCA**C**GAATGGAACAATC-3'

Apa I

10 S187A 5'-GGCTGACCCAGGGGCC**C**AACCACACCAAGC-3' (SEQ ID NO: 53)
5'-GCTTGGTGTGGTTGGGCCCTGGGTCAGCC-3'

Bcl I

D197N 5'-CTCCAGTTATT**AAC**GTGATCATTCCAGAAGG-3' (SEQ ID NO: 52)
15 5'-CCTTCTGGAATGATCAC**GTT**AATAACTGGAG-3'

Nco I

T214L 5'-CACTTTGGACCATGG**TC**TTTGTACTGCTTTCG-3' (SEQ ID NO: 54)
5'-CGAAAGCAGTACAAA**GA**CCATGGTCCAAAGTG-3'

20

Avr II

E222T 5'-GCTTTCGAAGACTCT**ACCC**TAGGTGACGACGTTG-3'
(SEQ ID NO: 55)
5'-CAACGTCGTCACCTA**GGGT**AGAGTCTTCGAAAGC-3'

25

V227A 5'-GGTGACGACG**C**TGAAGCTAACTTCAC-3' (SEQ ID NO: 56)
5'-GTGAAGTTAGCTTCA**G**CGTCGTCACC-3'

Sac II

30 L234V 5'-CTAACTTCACCGCGG**TG**TTCGCTCCAG-3' (SEQ ID NO: 57)
5'-CTGGAGCGAACA**CC**GCGGTGAAGTTAG-3'

A238P 5'-GCTTTGTTCGCTCCA**C**CTATTAGAGCTAGATTGG-3'
(SEQ ID NO: 58)
35 5'-CCAATCTAGCTCTAATAG**G**TGGAGCGAACAAAGC-3'

Hpa I

T251N 5'-GCCAGGTGTTAACTTG**AC**TGACGAAG-3' (SEQ ID NO: 59)
5'-TTCGTCAGTCAA**GT**TAACACCTGGC-3'
40

Aat II

Y259N 5'-GACGAAGACGTCGTT**A**ACTTGATGGAC-3' (SEQ ID NO: 60)
5'-GTCCATCAAGTT**A**ACGACGTCTTCGTC-3'

45

Asp I

E267D 5'-GTCCATTCGACACTGT**C**GCTAGAACTTC-3' (SEQ ID NO: 61)
5'-GAAGTTCTAGC**G**ACAGTGTCGAATGGAC-3'

40

E277Q   5'-CTGACGCTACTCAGCTGTCTCCATTC-3' (SEQ ID NO: 62)
       5'-GAATGGAGACAGCTGAGTAGCGTCAG-3'

5 A283D   5'-GTCTCCATTCTGTG**A**TTTGTTCACTCAC-3' (SEQ ID NO: 63)
       5'-GTGAGTGAACAAA**T**CACAGAATGGAGAC-3'

Ksp I

H287A   5'-GCTTTGTTCA***CCGCG***GACGAATGGAG-3' (SEQ ID NO: 64)
10   5'-CTCCATTCGT***CCGCG***GTGAACAAAGC-3'

Bam HI

R291I   5'-CACGACGAAT***GGATC***CAATACGACTAC-3' (SEQ ID NO: 65)
      5'-GTAGTCGTATT***GGA***TC***C***ATTCGTCGTG-3'
15

Bsi WI

Q292A   5'-GACGAATGGAGA***GCG***TACGACTACTTG-3' (SEQ ID NO: 66)
      5'-CAAGTAGT***CGTACGC***TCTCCATTCGTC-3'

Hpa I

20 A320V   5'-GGTGTTGGTTTC***GT***TAACGAATTGATTGC-3' (SEQ ID NO: 67)
      5'-GCAATCAATTC***GTTAA***CGAAACCAACACC-3'

(Bgl II)

25 R329H   5'-GCTAGATTGACT***CAC***TCTCCAGTTCAAG-3' (SEQ ID NO: 68)
      5'-CTTGAACTGGAGA***GTG***AGTCAATCTAGC-3'

Eco RV

S364T   5'-CTCACGACAAC**A**CTATG***ATA***T***C***TATTTTCTTC-3' (SEQ ID NO: 69)
30   5'-GAAGAAAATA***GATAT***CATAG**T**GTTGTCGTGAG-3'

Nco I

I366V   5'-CGACAACT***CCATGG***TTTCTATTTTCTTCGC-3' (SEQ ID NO: 70)
      5'-GCGAAGAAAATAGAAA***CCATGG***AGTTGTCG-3'
35

Kpn I

A379K   5'-GTACAAC***GGTAC***C***AAG***CCATTGTCTAC-3' (SEQ ID NO: 71)
      5'-GTAGACAATGG***CTTG***G***TACC***GTTGTAC-3'

40 S396A   5'-CTGACGGTTAC**G**CTGCTTCTTGGAC-3' (SEQ ID NO: 72)
      5'-GTCCAAGAAGCAG**C**GTAACCGTCAG-3'

G404A   5'-CTGTTCCATTCG**C**TGCTAGAGCTTAC-3' (SEQ ID NO: 73)
      5'-GTAAGCTCTAGCA**G**CGAATGGAACAG-3'
45

Q415E   5'-GATGCAATGT**G**AAGCTGAAAAGGAACC-3' (SEQ ID NO: 74)
      5'-GGTTCCTTTTCAGCTT**C**ACATTGCATC-3'

Sal I

A437G   5'-CACGGTTGTG**GT***GTC*GA**C**AAGTTGGG-3' (SEQ ID NO: 75)
        5'-CCCAACTT*GTCGA*C**A**CCACAACCGTG-3'

Mun I

A463E   5'-GATCTGGTGG*CAAT*TGGG**AG**GAATGTTTCG-3' (SEQ ID NO: 76)
        5'-CGAAACATTC**CT**CC*CAA*TT**G**CCACCAGATC-3'

and, accordingly, for other mutations.

The temperature optimum of the purified phytases, expressed in Saccharomyces cerevisiae (Example 7), was determined as outlined in Example 9. Table 5 shows the effect of each mutation introduced on the stability of consensus phytase-1.

Table 5

Stability effect of the individual amino acid replacements in consensus phytase-1

+ or - means a positive, respectively, negative effect on the protein stability up to 1°C, ++ and -- means a positive, respectively, negative effect on the protein stability between 1 and 3°C; the numbers 10 or 11 in parentheses indicate the consensus phytase sequence that suggested the amino acid replacement.

stabilizing		neutral		destabilizing	
mutation	effect	mutation	ef-fect	mutation	effect
E58A (10)	+	D69A	±	Y54F (10)	-
D69K (11)	+	D70G (10)	±	V73I	-
D197N (10)	+	N134Q (10)	±	A94K (10)	-
T214L (10)	+ +	G186H	±	A101R (11)	-
E222T (11)	+ +	S187A (10)	±	K153N (11)	-
E267D (10)	+	T214V	±	I158V (10)	- -
R291I	+	T251N (10)	±	G203A	- -
R329H (10)	+	Y259N (10)	±	G205S	-
S364T (10)	+ +	A283D (10)	±	A217V	-
A379K (11)	+	A320V (10)	±	V227A (11)	- -
G404A (10)	+ +	K445T	±	L234V (10)	-
		A463E (10)	±	A238P (10)	- -
				E277Q (10)	-
				H287A (11)	-
				Q292A	-
				I366V (10)	-
				S396A (10)	- -
				Q415E (11)	-
				A437G (10)	- -
				E451R	- -

We combined eight positive mutations (E58A, D197N, E267D, R291I, R329H, S364T, A379K, G404A) in consensus phytase-1 thermo[8], using the primers and the technique mentioned above in this example. Furthermore, the mutations Q50T and/or K91A were introduced which mainly influence the catalytic characteristics of phytase (see patent applications EP 897010 and EP 897985, as well as Example 9). The DNA and amino acid sequence of the resulting phytase (consensus phytase-1-thermo[8]-Q50T-K91A) are shown in Figure 7. In this way, the temperature optimum and the melting point of the consensus phytase were increased by 7°C (Figures 15, 16, 17).

In a further consensus protein, we combined eleven positive mutations (E58A, D69K, D197N, T214L, E222T, E267D, R291I, R329H, S364T, A379K, G404A) in consensus phytase-1 thermo[11]. Furthermore, the mutations Q50T and/or K91A were

introduced. In this way, the melting temperature was increased by another 3-4°C when compared to consensus phytase-1 thermo[8].

Using the results of Table 5, we further improved the thermostability of consensus phytase-10 by the back mutations
5 K94A, V158I, and A396S, the reverse of which (A94K, I158V, and S396A) revealed a strong negative influence on the stability of consensus phytase-1. The resulting protein was called consensus phytase-10-thermo[3]. SEQ ID NO: 26 plus the three mutations K94A, V158I, and A396S. Furthermore, we introduced the mutations
10 Q50T and K91A that mainly influence the catalytic characteristics of consensus phytase (see patent applications EP 897010 and EP 897985, as well as Example 9 and Figures 14 and 15). The resulting DNA and amino acid sequence are shown in Figure 8. The optimized phytase showed a 4°C higher temperature
15 optimum and melting point than consensus phytase-10 (Figures 12 and 13). Furthermore, the phytase has also a strongly increased specific activity with phytate as substrate of 250 U/mg at pH 5.5 (Figure 14).

In a still further consensus protein, two additional
20 mutations were introduced into consensus phytase-10 thermo[3] (E222T, G437A) which yielded consensus phytase-10 thermo[5]. Furthermore, the mutations Q50T and/or K91A were introduced. In this way, the melting temperature was increased by another 1-2°C when compared to consensus phytase-10 thermo[3].

25

Example 4

Stabilization of the phytase of A. fumigatus ATCC 13073 by replacement of amino acid residues with the corresponding consensus phytase-1 and/or consensus phytase-10 residues

At six amino acid sequence positions where A. fumigatus 13073 phytase is the only or nearly the only phytase in the alignment of Figure 1 that does not contain the corresponding consensus phytase amino acid residue, the non-consensus amino acid residue was replaced by the consensus one. The following amino acids were substituted in A. fumigatus 13073 phytase, containing additionally the Q51(24)T substitution (influencing the catalytic properties and corresponding to the Q50T substitution in the consensus phytases) and the signal sequence of A. terreus cbs116.46 phytase (see European Patent Application No. 0897010, and Figure 9): F55(28)Y, V100(73)I, F114(87)Y, A243(220)L, S265(242)P, N294(282)D. The numbers in parentheses refer to the numbering in Figure 1.

In a second round, four of the seven stabilizing amino acid exchanges (E58A, R329H, S364T, G404A) identified in consensus phytase-10 and tested as single mutations in consensus phytase-1 (Table 5) were additionally introduced into the A. fumigatus alpha-mutant. Furthermore, the amino acid replacement S154N, shown to reduce the protease susceptibility of the phytase, was introduced.

The mutations were introduced as described in Example 3 (see Table 6) and expressed as described in Examples 6 to 8. The resulting A. fumigatus 13073 phytase variants were called alpha-mutant (i.e. the A. fumigatus ATCC 13073 phytase with the substitutions Q24T, F28Y, V73I, F87Y, A220L, S242P, N282D) and ''optimized'' alpha-mutant (i.e. the A. fumigatus alpha-mutant

having the additional substitutions E59A-S154N-R329H-S364T-G404A). K92A is an additional preferred mutation.

The temperature optimum (60°C, Figure 20) and the melting temperature (67.0°C, Figure 19) of the A. fumigatus 13073 alpha-mutant phytase were increased by 5-7°C in comparison to the values of the wild-type phytase (temperature optimum: 55°C, Tm: 60°C). The five additional amino acid replacements further increased the temperature optimum by 3°C (Figure 20).

Table 6

Mutagenesis primers for the stabilization of A. fumigatus ATCC 13073 phytase

Mutation	Primer
F55Y	5'-CACGTACTCGCCA**TAC**TTTTCGCTCGAG-3' (SEQ ID NO: 77) 5'-CTCGAGCGAAAAGT**ATG**GCGAGTACGTG-3'
	(*Xho* I)
E58A	5'-CCATACTTTTCG*CTCGC*GGACGAGCTGTCCGTG-3' (SEQ ID NO: 78) 5'-CACGGACAGCTCGTCC*GCGAG*CGAAAAGTAGG-3'
V100I	5'-GTATAAGAAGCTT**ATT**ACGGCGATCCAGGCC-3' (SEQ ID NO: 79) 5'-GGCCTGGATCGCCGTAA**TAA**GCTTCTTATAC-3'
F114Y	5'-CTTCAAGGGCAAG**TAC**GCCTTTTTGAAGACG-3' (SEQ ID NO: 80) 5'-CGTCTTCAAAAAGGC**GTA**CTTGCCCTTGAAG-3'
A243L	5'-CATCCGAGCTCGC**CTC**GAGAAGCATCTTC-3' (SEQ ID NO: 81) 5'-GAAGATGCTTCTC**GAG**GCGAGCTCGGATG-3'
S265P	5'-CTAATGGA TGTGT**CCG**TTTGATACGGTAG-3' (SEQ ID NO: 82) 5'-CTACCGTATCAAA**CGG**ACACATGTCCATTAG-3'
N294D	5'-GTGGAAGAAGTA**CGA**CTACCTTCAGTC-3' (SEQ ID NO: 83) 5'-GACTGAAGGTAG**TCG**TACTTCTTCCAC-3'

46

(*Mlu* I)

R329H   5'-GCCCGGTTGA*CGC***A**TTCGCCAGTGCAGG-3' (SEQ ID NO: 84)
         5'-CCTGCACTGGCGAA*TGCGT*CAACCGGGC-3'

5

Nco I

S364T   5'-CACACGACAACA*CCATGG*TTTCCATCTTC-3' (SEQ ID NO: 85)
         5'-GAAGATGGAAA*CCATG***G**TGTTGTCGTGTG-3'

(*Bss* HI)

10 G404A   5'-GTGGTGCCTTTCG*C***C**GCGC*G*AGCCTACTTC-3' (SEQ ID NO: 86)
         5'-GAAGTAGGCTC*GCGCG***G**CGAAAGGCACCAC-3'

Example 5

Introduction of the active site amino acid residues of A. niger NRRL 3135 phytase into consensus phytase-1

We used the crystal structure of Aspergillus niger NRRL 3135 phytase to define all active site amino acid residues (see Example 1, and EP 897010). Using the alignment of Figure 1, we replaced the following active site residues and additionally the non-identical adjacent ones of consensus phytase-1 by those of A. niger phytase:

S89D, S92G, A94K, D164S, P201S, G203A, G205S, H212P, G224A, D226T, E255T, D256E, V258T, P265S, Q292H, G300K, Y305H, A314T, S364G, M365I, A397S, S398A, G404A, and A405S.

The new consensus phytase-7 protein sequence was backtranslated into a DNA sequence (Figure 10) as described in Example 1. The corresponding gene (fcp7) was generated as described in Example 1 using the following oligonucleotide mixes:

Mix 1.7:   CP-1, CP-2, CP-3, CP-4.7, CP-5.7, CP-6, CP-7, CP-8.7, CP-9, CP-10.7

Mix 2.7:   CP-9, CP-10.7, CP-11.7, CP-12.7, CP-13.7, CP-14.7, CP-15.7, CP-16, CP-17.7, CP-18.7, CP-19.7, CP-20, CP-21, CP-22.

The DNA sequences of the oligonucleotides are indicated in Figure 10. The newly synthesized oligonucleotides are additionally marked by the number 7. After assembling of the

5 oligonucleotides using the same PCR primers as mentioned in Example 1, the gene was cloned into an expression vector as described in Examples 6-8.

The pH-profile of the enzyme determined after expression in H. polymorpha and purification was very similar to that of A.

10 niger phytase (see Figure 18).

Example 6

Expression of the consensus phytase genes in Hansenula polymorpha

15 The phytase expression vectors used to transform H. polymorpha RB11 [Gellissen, G., Hollenberg, C. P., Janowicz, Z. A. (1994) Gene expression in methylotrophic yeasts, in Smith, A. (ed.) Gene expression in recombinant microorganisms. Dekker, New York, pp. 395-439] were constructed by inserting the Eco RI fragment

20 of pBsk-fcp or variants thereof into the multiple cloning site of the H. polymorpha expression vector pFPMT121, which is based on an ura3 selection marker from S. cerevisiae, a formate dehy- drogenase (FMD) promoter element and a methanol oxidase (MO) terminator element from H. polymorpha. The 5′ end of the fcp

25 gene is fused to the FMD promoter, the 3′ end to the MOX termi- nator (Gellissen et al., Appl. Microbiol. Biotechnol. 46, 46-54, 1996; EP 299108). The resulting expression vectors are desig- nated pFPMTfcp, pFPMTfcp10, and pFPMTfcp7.

The constructed plasmids were propagated in E. coli.

30 Plasmid DNA was purified using standard state of the art procedures. The expression plasmids were transformed into the H.

polymorpha strain RB11 deficient in orotidine-5'-phosphate decarboxylase (ura3) using the procedure for preparation of competent cells and for transformation of yeast as described in Gellissen et al. (1996). Each transformation mixture was plated

5 on YNB medium (0.14% w/v Difco YNB and 0.5% ammonium sulfate) containing 2% glucose and 1.8% agar, and incubated at 37 °C. After 4 to 5 days individual transformant colonies were picked and grown in the liquid medium described above for 2 days at 37 °C. Subsequently, an aliquot of this culture was used to

10 inoculate fresh vials with YNB-medium containing 2% glucose. After seven further passages in selective medium, the expression vector had integrated into the yeast genome in multimeric form. Subsequently, mitotically stable transformants were obtained by two additional cultivation steps in 3 ml non-selective liquid

15 medium (YPD, 2% glucose, 10 g/l yeast extract, and 20 g/l peptone). In order to obtain genetically homogeneous recombinant strains, an aliquot from the last stabilization culture was plated on a selective plate. Single colonies were isolated for analysis of phytase expression in YNB containing 2% glycerol

20 instead of glucose to derepress the FMD promoter. Purification of the consensus phytases was done as described in Example 7.

Example 7

Expression of the consensus phytase genes in Saccharomyces

25 cerevisiae and purification of the phytases from the culture supernatant

The consensus phytase genes were isolated from the corresponding Bluescript-plasmid (pBsk-fcp, pBSK-fcp10, pBsk-

30 fcp7) and ligated into the Eco RI sites of the expression cassette of the Saccharomyces cerevisiae expression vector pYES2

(Invitrogen, San Diego, CA, USA) or subcloned between the shortened GAPFL (glyceraldhyde-3-phosphate dehydrogenase) promoter and the pho5 terminator as described by Janes et al., Curr. Genet. 18, 97-103. The correct orientation of the gene was

5 checked by PCR. Transformation of S. cerevisiae strains, e.g. INVSc1 (Invitrogen, San Diego, CA, USA), was done according to Hinnen et al., Proc. Natl. Acad. Sci. USA 75, 1929-1933 (1978). Single colonies harboring the phytase gene under the control of the GAPFL promoter were picked and cultivated in 5 ml selection

10 medium [SD-uracil; Sherman, J. P., Finck, G. R. & Hicks, J. B. (1986) Laboratory course manual for methods in yeast genetics. Cold Spring Harbor University] at 30°C under vigorous shaking (250 rpm) for one day. The preculture was then added to 500 ml YPD medium (Sherman et al., 1986) and grown under the same

15 conditions. Induction of the gal1 promoter was done according to the manufacturer's instructions. After four days of incubation, the cell broth was centrifuged (7000 rpm, GS3 rotor, 15 min, 5°C) to remove the cells, and the supernatant was concentrated by way of ultrafiltration in Amicon 8400 cells (PM30 membranes;

20 Grace AG, Wallizeller, Switzerland) and ultrafree-15 centrifugal filter devices (Biomax-30K, Millipore, Bedford, MA, USA). The concentrate (10 ml) was desalted on a 40 ml Sephadex G25 Superfine column (Pharmacia Biotech, Freiburg, Germany), with 10 mM sodium acetate, pH 5.0, serving as elution buffer. The

25 desalted sample was brought to 2 M $(NH_4)_2SO_4$ and directly loaded onto a 1 ml Butyl Sepharose 4 Fast Flow hydrophobic interaction chromatography column (Pharmacia Biotech, Feiburg, Germany) which was eluted with a linear gradient from 2 M to 0 M $(NH_4)_2SO_4$ in 10 mM sodium acetate, pH 5.0. Phytase was eluted in the

30 breakthrough, concentrated and loaded on a 120 ml Sephacryl S-300 gel permeation chromatography column (Pharmacia Biotech,

Freiburg, Germany). Consensus phytases -1, -7 and -10 eluted as a homogeneous symmetrical peak and were shown by SDS-PAGE to be approx. 95% pure.

5   Example 8

Expression of the consensus phytase genes in Aspergillus niger

The Bluescript-plasmids pBsk-fcp, pBsk-fcp10, and pBsk-fcp7 were used as template for the introduction of a Bsp HI-site

10   upstream of the start codon of the genes and an Eco RV-site downstream of the stop codon. The ExpandTM High Fidelity PCR Kit (Boehringer Mannheim, Mannheim, Germany) was used with the following primers:

15   Primer Asp-1:

Bsp HI

5'-TATA*TCATGA*GCGTGTTCGTCGTGCTACTGTTC-3' (SEQ ID NO: 87)

Primer Asp-2 used for cloning of fcp and fcp7:

20

Eco RV

3'-ACCCGACTTACAAAGCGAATT*CTATAG*ATATAT-5' (SEQ ID NO: 88)

Primer Asp-3 used for cloning of fcp10:

25

Eco RV

3'-ACCCTTCTTACAAAGCGAATT*CTATAG*ATATAT-5' (SEQ ID NO: 89)

The reaction was performed as described by the supplier.

30 The PCR-amplified fcp-genes had a new Bsp HI site at the start codon, introduced by primer Asp-1, which resulted in a

replacement of the second amino acid residue glycine by serine. Subsequently, the DNA-fragment was digested with Bsp HI and Eco RV and ligated into the Nco I site downstream of the glucoamylase promoter of Aspergillus niger (glaA) and the Eco RV

5 site upstream of the Aspergillus nidulans tryptophan C terminator (trpC) (Mullaney et al., 1985). After this cloning step, the genes were sequenced to detect possible errors introduced by PCR. The resulting expression plasmids, which basically correspond to the pGLAC vector as described in Example

10 9 of EP 684313, contained the orotidine-5'-phosphate decarboxylase gene (pyr4) of Neurospora crassa as a selection marker. Transformation of Aspergillus niger and expression of the consensus phytase genes was done as described in EP 684313. The consensus phytases were purified as described in Example 7.

15

Example 9

Determination of phytase activity and of the pH and temperature optima

This example relates i.a. to the determination of phytase

20 activity and of the temperature optimum. Various phytases have been tested.

The phytase of Aspergillus niger NRRL 3135 was prepared as described in EP 420358 and by van Hartingsveldt et al. (Gene 127, 87-94, 1993).

25 The phytases of Aspergillus fumigatus ATCC 13073, Aspergillus terreus 9A-1, Aspergillus terreus cbs116.46, Emericella nidulans, Myceliophthora thermophila, and Talaromyces thermophilus were prepared as described in EP-0897985 and in the references therein.

30 The remaining phytases tested were prepared as described herein.

Consensus phytase-1-thermo(8) designates a variant of consensus phytase-1, which further comprises the eight mutations which are underlined in the legend to Figure 5. Consensus phytase-1 is shown in Fig. 1 (SEQ ID NO: 14) without signal
5 peptide, and in Fig. 2 (SEQ ID NO: 16) with the signal peptide.

Phytase activity was determined basically as described by Mitchell et al. (1997). The activity was measured in an assay mixture containing 0.5% phytic acid ($\approx$5 mM) in 200 mM sodium acetate, pH 5.0. After 15 min of incubation at 37°C, the reac-
10 tion was stopped by addition of an equal volume of 15% trichloroacetic acid. The liberated inorganic phosphate was quantified by mixing 100 μl of the assay mixture with 900 μl H_2O and 1 ml of 0.6 M H_2SO_4, 2% ascorbic acid and 0.5% ammonium molybdate. Standard solutions of potassium phosphate were used as refer-
15 ence. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol phosphate per minute at 37°C. The protein concentration was determined using the enzyme extinction coefficient at 280 nm calculated according to Pace et al. [Pace N. C., Vajdos, F., Fee, L., Grimsley, G. & Gray, T. (1995) How
20 to measure and predict the molar absorption coefficient of a protein. Prot. Sci. 4, 2411-2423]: 1 absorption unit (1 OD) at 280 nm corresponds to 1.101 mg/ml of consensus phytase-1, 1.068 mg/ml of consensus phytase-7, and 1.039 mg/ml of consensus phytase-10.

25 In case of pH-optimum curves, the purified enzymes were diluted in 10 mM sodium acetate, pH 5.0. Incubations were started by mixing aliquots of the diluted protein with an equal volume of 1% phytic acid ($\approx$10 mM) in a series of different buffers: 0.4 M glycine/HCl, pH 2.5; 0.4 M acetate/NaOH, pH 3.0,
30 3.5, 4.0, 4.5, 5.0, 5.5; 0.4 M imidazole/HCl, pH 6.0, 6.5; 0.4 M Tris/HCl pH 7.0, 7.5, 8.0, 8.5, 9.0. Control experiments showed

that pH was only slightly affected by the mixing step. Incubations were performed for 15 min at 37°C as described above.

For determination of the substrate specificities of the 5 phytases, phytic acid in the assay mixture was replaced by 5 mM concentrations of the respective phosphate compounds. Besides, the activity tests were performed as described above.

For determination of the temperature optimum, enzyme (100 μl) and substrate solution (100 μl) were pre-incubated for 5 min 10 at the given temperature. The reaction was started by addition of the substrate solution to the enzyme. After 15 min of incubation, the reaction was stopped with trichloroacetic acid, and the amount of phosphate released was determined.

The pH-optimum of consensus phytase-1 was around pH 6.0- 15 6.5 (70 U/mg). Introduction of the Q50T mutation shifted the pH-optimum to pH 6.0 (130 U/mg). Introduction of the K91A mutation further shifted the pH optimum into the more acidic pH- range. Comparable effects of the Q50T and K91A mutations were also observed for consensus phytase-10 and for further 20 stabilized consensus phytase variants (Figures 14 and 15).

Consensus phytase-7, which was constructed to transfer the catalytic characteristics of A. niger NRRL 3135 phytase to consensus phytase-1, had a pH-profile very similar to that of A. niger NRRL 3135 phytase (see Figure 18). The substrate 25 specificity also resembled more that of A. niger NRRL 3135 phytase than that of consensus phytase-1.

The temperature optimum of consensus phytase-1 (71°C) was 16-26°C higher than the temperature optima of the wild-type phytases (45-55°C, Table 7) that were used to calculate the 30 consensus sequence. The improved consensus phytase-10 showed a further increase of its temperature optimum to 80°C (Figure 13).

The temperature optimum of consensus phytase-1-thermo[8] was found to be in the same range (78°C) when using the supernatant of an overproducing S. cerevisiae strain. The highest temperature optimum reached of 82°C was determined for

5 consensus phytase-10-thermo[3]-Q50T-K91A. Table 7

Temperature optima and Tm-values of consensus phytase and of the phytases from A. fumigatus, A. niger, E. nidulans, and M. thermophila.

10

The determination of the temperature optimum was performed as described in Example 9. The Tm-values were determined by differential scanning calorimetry as described in Example 10.

Phytase	Optimum temperature (°C)	Tm (°C)
Aspergillus niger NRRL 3135	55	63.3
Aspergillus fumigatus ATCC 13073	55	62.5
Aspergillus terreus 9A-1	49	57.5
Aspergillus terreus cbs116.46	45	58.5
Emericella nidulans	45	55.7
Myceliophthora thermophila	55	-
Talaromyces thermophilus	45	-
Consensus phytase-10-thermo[5]-Q50T-K91A	-	90.4
Consensus-phytase-10-thermo[3]-Q50T-K91A	82	89.3

Consensus-phytase-10-thermo[3]-Q50T	82	88.6
Consensus-phytase-10	80	85.4
Consensus phytase-1-thermo[11]-Q50T-K91A	-	88.0
Consensus phytase-1-thermo[11]-Q50T	-	88.5
Consensus-phytase-1-thermo[8]-Q50T-K91A	-	85.7
Consensus-phytase-1-thermo[8]-Q50T	78	84.7
Consensus-phytase-1-thermo[8]	81	-
Consensus-phytase-1-thermo[3]	75	-
Consensus-phytase-1-Q50T	-	78.9
Consensus-phytase-1	71	78.1
Aspergillus fumigatus α-mutant Q51T	60	67.0
Aspergillus fumigatus α-mutant, plus mutations E59A, S154N, R329H, S364T, G404A	63	-
Aspergillus fumigatus ''optimized'' alpha-mutant, plus mutation K92A	63	-

Example 10

Determination of the melting temperature by differential scanning calorimetry (DSC)

5   In order to determine the unfolding temperature of the phytases, differential scanning calorimetry was applied as described by Brugger et al., 1997 [Brugger, R., Mascarello, F., Augem, S., van Loon, A. P. G. M. & Wyss, M. (1997). Thermal denaturation of phytases and pH 2.5 acid phosphatase studied by 10 differential scanning calorimetry. In The Biochemistry of phytate and phytase (eds. Rasmussen, S.K.; Raboy, V.; Dalbøge, H. and Loewus, F.; Kluwer Academic Publishers, Dordrecht, the Netherlands]. Solutions of 50-60 mg/ml of homogeneous phytase were used for the tests. A constant heating rate of 10°C/min was applied up to 90-95°C.

15 plied up to 90-95°C.

The determined melting points confirm the results obtained for the temperature optima (Table 7). The most stable consensus phytase designed so far is consensus phytase-10-thermo[3]-Q50T-K91A showing a melting temperature under the chosen conditions 20 of 89.3°C. This is 26.0 to 33.6°C higher than the melting temperature of the wild-type phytases used.

Example 11

Transfer of basidiomycete phytase active site into 25 consensus phytase-10-thermo[3]-Q50T-K91A

As described previously (Example 5), mutations derived from the basidiomycete phytase active sites were introduced into consensus phytase-10. The following five constructs a) to e) were prepared:

30   a)   The construct called consensus phytase-12, and it comprises a selected number of active site residues of the

basidio consensus sequence. Its amino acid sequence is shown in Fig. 21 (the first 26 amino acids form the signal peptide; positions differing from consensus phytase-10-thermo[3]-Q50T-K91A are underlined);

5   b) a cluster of mutations (Cluster II) was transferred to the consensus phytase-1 and -10 sequences, viz.: S80Q, Y86F, S90G, K91A, S92A, K93T, A94R, Y95I;

c) in a similar way, another cluster of mutations (Cluster III) was transferred, viz.: T129V, E133A, Q134N, M136S, V137S, N138Q, S139A;

d) in a similar way, a further cluster of mutations (Cluster IV) was transferred, viz.: A168D, E171T, K172N, F173W;

e) and finally, a further cluster of mutations (Cluster V) was transferred, viz.: Q297G, S298D, G300D, Y305T.

15   These constructs were expressed as described in Examples 6 to 8.

Example 12

Phytase alignment using GAP

20   The phytases described herein - i.e. the amino acid sequences as well as the corresponding DNA sequences - were aligned against each other. Also some other phytases were correspondingly aligned, viz. the following:

- the consensus phytase-1 described in EP 897985;

25 - the phytase derived from Aspergillus niger (ficuum) NRRL 3135 (A. niger NRRL3135) described in EP 420358;

- the phytases derived from Aspergillus fumigatus ATCC 13073 (A. fumigatus 13073); Aspergillus fumigatus ATCC 32239 (A. fumigatus 32239); Aspergillus terreus cbs116.46 (A.terreus cbs);

30 Emericella nidulans (E. nidulans); and Talaromyces thermophilus (T. thermophilus) - all described in EP 897010;

- the phytases derived from Myceliophthora thermophila (M. thermophila); and Aspergillus terreus 9-A1 (A. terreus 9-A1) - both described in EP 684313;

- the phytase derived from Thermomyces lanuginosus (T. 5 lanuginosus) described in WO 9735017 (PCT/US97/04559);

- the phytases derived from Agrocybe pediades (A. pediades), Paxillus involutus 1 and 2 (P. involutus phyA1 and phyA2); and Trametes pubescens (T. pubescens) - all described in WO 98/28409; and

10 - the phytase derived from Peniophora lycii (P. lycii) described in WO 98/28408.

For the alignments, the program GAP was used with the settings as described above.

For polypeptide comparisons, the signal peptides were 15 included with the exception of comparisons with consensus phytase-11.

The results of the amino acid sequence comparisons are shown in Table 8 below. The first number in each cell is the amino acid similarity, the second number is the amino acid 20 identity.

For DNA sequence comparisons, the signal sequence was always included. The results are shown in Table 9 below.

This invention comprises e.g. the following embodiments (A) to (J) that are described below.

25 In these embodiments, when determining % identity or % similarity at the amino acid level for another phytase, its amino acid sequence is aligned with the reference sequence (e.g. in embodiment (A) the consensus phytase-10 amino acid sequence), using an alignment program such as GAP referred to above. 30 Percentage identity, as well as percentage similarity, is

calculated by the program. The amino acid sequence of the other phytase may or may not include the signal peptide.

When determining % identity on the DNA level for another phytase-encoding DNA, this DNA sequence is aligned with the
5 reference sequence [e.g. in embodiment (A) nucleotides 12-1412 of SEQ ID NO: 25 (the DNA sequence of consensus phytase-10 (Fcp10) as shown in Fig. 5], using an alignment program such as GAP referred to above. Percentage identity is calculated by the program. The DNA sequence encoding the other phytase can be a
10 genomic DNA sequence including introns, or it can be a cDNA sequence. It may or may not include the signal peptide-encoding part.

When determining hybridization, the probe to be used is the specified DNA sequence [e.g. in embodiment (A) nucleotides
15 12-1412 of SEQ ID NO: 25 (the DNA sequence of consensus phytase-10 (Fcp10) as shown in Fig. 5)]. The DNA sequence encoding the other phytase can be a genomic DNA sample which contains a phytase-encoding DNA-sequence; a purified genomic DNA sequence (purified with respect to the phytase-encoding DNA sequence); or
20 it can be a phytase-encoding cDNA sequence, preferably purified or amplified, e.g. PCR-amplified. The phytase-encoding DNA, whatever type, may or may not include the signal peptide-encoding part. Suitable hybridization conditions are referred to above.

25   The term ''DNA sequence'' includes such fragments or parts of the herein exemplified DNA sequences, as long as they are capable of encoding an active enzyme (e.g. phytase).

The term ''amino acid sequence'' includes such fragments or parts of the herein exemplified amino acid sequences, as long
30 as they are enzymatically active (e.g. displaying phytase activity).

(A) Phytases and corresponding DNA sequences related to consensus phytase-10 (CP10, Fcp 10)

A phytase that comprises an amino acid sequence which is at least 93.80%; or at least 94, 94.5, 95, 95.5, 96, 96.5, 97, 5 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino acids 1-467 of consensus phytase-10 (Fcp10) as shown in Fig. 5.

A phytase that comprises an amino acid sequence which is at least 95.09%; or at least 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% similar to the sequence of amino acids 1-467 of 10 consensus phytase-10.

A phytase which is encoded by a DNA sequence which is at least 95.88%; or at least 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to nucleotides 12-1412 of the DNA sequence of consensus phytase-10 (Fcp10) as shown in Fig. 5.

15   A DNA sequence which encodes a phytase and which (i) is at least 95.88%; or at least 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical; or (ii) hybridizes under low, or medium, medium/high, high, or very high stringency conditions to nucleotides 12-1412 of the DNA sequence of consensus phytase-10 20 (Fcp10) as shown in Fig. 5. A suitable negative control is DNA encoding consensus phytase-1. A suitable positive control is DNA encoding any of CP10, CP10-thermo[3]-Q50T, K91A, CP1-thermo[8], CP1-thermo[8]Q50T,K91A.

A DNA sequence which encodes a phytase comprising an amino 25 acid sequence which is at least 93.80%; or at least 94, 94.5, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino acids 1-467 of consensus phytase-10 (Fcp10) as shown in Fig. 5.

(B) Phytases and corresponding DNA sequences related to
consensus phytase-10-thermo[3]-Q50T-K91A

A phytase which comprises an amino acid sequence which is
at least 93.37%; or at least 93.5, 94, 94.5, 95, 95.5, 96, 96.5,
5 97, 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino
acids 1-467 of consensus phytase-10-thermo[3]-Q50T-K91A as shown
in Fig. 8.

A phytase which comprises an amino acid sequence which is
at least 94.66%; or at least 95.0, 95.5, 96, 96.5, 97, 97.5, 98,
10 98.5, 99, 99.5% similar to the sequence of amino acids 1-467 of
consensus phytase-10-thermo[3]-Q50T-K91A as shown in Fig. 8.

A phytase which is encoded by a DNA sequence which is at
least 95.88%; or at least 96, 96.5, 97, 97.5, 98, 98.5, 99,
99.5% identical to nucleotides 12-1412 of the DNA sequence of
15 consensus phytase-10-thermo[3]-Q50T-K91A as shown in Fig. 8.

A DNA sequence which encodes a phytase and which (i) is at
least 95.88%; or at least 96, 96.5, 97, 97.5, 98, 98.5, 99,
99.5% identical; or (ii) hybridizes under low, or medium,
medium/high, high, or very high stringency conditions to
20 nucleotides 12-1412 of the DNA sequence of consensus phytase-10-
thermo[3]-Q50T-K91A as shown in Fig. 8. A suitable negative
control is DNA encoding consensus phytase-1. A suitable positive
control is DNA encoding any of CP10, CP10-thermo[3]-Q50T-K91A,
CP1-thermo[8], or CP1-thermo[8]-Q50T-K91A.

25   A DNA sequence which encodes a phytase comprising an amino
acid sequence which is at least 93.37%; or at least 93.5, 94,
94.5, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5%
identical to the sequence of amino acids 1-467 of consensus
phytase-10-thermo[3]-Q50T-K91A as shown in Fig. 8.

30

<u>(C) Phytases and corresponding DNA sequences related to consensus phytase-1-thermo[8]</u>

A phytase which comprises an amino acid sequence which is at least 98.30%; or at least 98.5, 99, 99.5% identical to the sequence of amino acids 1-467 of consensus phytase-1-thermo[8] (as shown in Fig. 7; backmutations T50Q and A91K to be added).

A phytase which comprises an amino acid sequence which is at least 98.51%; or at least 99, 99.5% similar to the sequence of amino acids 1-467 of consensus phytase-1-thermo[8] (as shown in Fig. 7; backmutations T50Q and A91K to be added).

A phytase which is encoded by a DNA sequence which is at least 98.73%; or at least 99, 99.5% identical to nucleotides 1-1407 of the DNA sequence of consensus phytase-1-thermo[8] (as shown in Fig. 7; backmutations T50Q and A91K to be added).

A DNA sequence which encodes a phytase and which (i) is at least 98.73%; or at least 99, 99.5% identical; or (ii) hybridizes under low, or medium, medium/high, high, or very high stringency conditions to nucleotides 1-1407 of the DNA sequence of consensus phytase-1-thermo[8] (as shown in Fig. 7; backmutations T50Q and A91K to be added). A suitable negative control is DNA encoding consensus phytase-1. A suitable positive control is DNA encoding any of CP1-thermo[8], CP1-thermo[8]-Q50T-K91A.

A DNA sequence which encodes a phytase comprising an amino acid sequence which is at least 98.30%; or at least 98.5, 99, 99.5% identical to the sequence of amino acids 1-467 of consensus phytase-1-thermo[8] (as shown in Fig. 7; backmutations T50Q and A91K to be added).

(D) Phytases and corresponding DNA sequences related to consensus phytase-1-thermo[8]-Q50T-K91A

A phytase which comprises an amino acid sequence which is at least 97.87%; or at least 98, 98.5, 99, 99.5% identical to

5 the sequence of amino acids 1-467 of consensus phytase-1-thermo[8]-Q50T-K91A as shown in Fig. 7.

A phytase which comprises an amino acid sequence which is at least 98.08%; or at least 98.5, 99, 99.5% similar to the sequence of amino acids 1-467 of consensus phytase-1-thermo[8]-

10 Q50T-K91A as shown in Fig. 7.

A phytase which is encoded by a DNA sequence which is at least 98.37%; or at least 98.5, 99, 99.5% identical to nucleotides 1-1407 of the DNA sequence of consensus phytase-1-thermo[8]-Q50T-K91A as shown in Fig. 7.

15 A DNA sequence which encodes a phytase and which (i) is at least 98.37%; or at least 98.5, 99, 99.5% identical; or (ii) hybridizes under low, or medium, medium/high, high, or very high stringency conditions to nucleotides 1-1407 of the DNA sequence of consensus phytase-1-thermo[8]-Q50T-K91A as shown in Fig. 7. A

20 suitable negative control is DNA encoding consensus phytase-1. A suitable positive control is DNA encoding any of CP1-thermo[8], CP1-thermo[8]-Q50T-K91A.

A DNA sequence which encodes a phytase comprising an amino acid sequence which is at least 97.87%; or at least 98, 98.5,

25 99, 99.5% identical to the sequence of amino acids 1-467 of consensus phytase 1-thermo[8]-Q50T-K91A as shown in Fig. 7.

(E) Phytases and corresponding DNA sequences related to consensus phytase-11

30 A phytase that comprises an amino acid sequence which is at least 90.71%; or at least 91, 91.5, 92, 92.5, 93, 93.5, 94,

94.5, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino acids 1-482 of consensus phytase-11 as shown in Fig. 6.

A phytase that comprises an amino acid sequence which is at least 92.07%; or at least 92.5, 93, 93.5, 94, 94.5, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% similar to the sequence of amino acids 1-482 of consensus phytase-11 as shown in Fig. 6.

A DNA sequence that encodes a phytase comprising an amino acid sequence which is at least 90.71%; or at least 91. 91.5, 92, 92.5, 93, 93.5, 94, 94.5, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino acids 1-482 of consensus phytase-11 as shown in Fig. 6.

(F) Phytases and corresponding DNA sequences related to A. fumigatus alpha-mutant

A phytase that comprises an amino acid sequence which is at least 97.17%; or at least 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino acids 1-467 of A. fumigatus alpha-mutant (phytase) as shown in Fig. 9.

A phytase that comprises an amino acid sequence that is at least 97.82%; or at least 98, 98.5, 99, 99.5% similar to the sequence of amino acids 1-467 of A. fumigatus alpha-mutant (phytase) as shown in Fig. 9.

A phytase which is encoded by a DNA sequence which is at least 96.13%; or at least 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to nucleotides 1-1401 of the DNA sequence of A. fumigatus ATCC 13073 alpha-mutant (phytase) as shown in Fig. 9.

A DNA sequence which encodes a phytase comprising an amino acid sequence which is at least 97.17%; or at least 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino acids 1-467

of A. fumigatus ATCC 13073 alpha-mutant (phytase) as shown in Fig. 9.

A DNA sequence which encodes a phytase and which (i) is at least 96.13%; or 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical; or (ii) hybridizes under low, or medium, medium/high, high, or very high stringency conditions to nucleotides 1-1401 of the DNA sequence of A. fumigatus ATCC 13073 alpha-mutant (phytase) shown in Fig. 9. A suitable negative control is DNA encoding A. fumigatus 13073 phytase. A suitable positive control is DNA encoding any one of the A. fumigatus ATCC 13073 alpha mutant phytase or the optimised alpha-mutant.

(G) Phytases and corresponding DNA sequences related to the optimized A. fumigatus alpha-mutant

A phytase that comprises an amino acid sequence that is at least 96.08%; or at least 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to the sequence of the phytase of the optimized A. fumigatus alpha-mutant.

A phytase that comprises an amino acid sequence that is at least 96.74%; or at least 97, 97.5, 98, 98.5, 99, 99.5% similar to the sequence of the phytase of the optimized A. fumigatus alpha-mutant.

A phytase which is encoded by a DNA sequence which is at least 95.63%; or at least 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to nucleotides 1-1401 of the DNA sequence encoding the optimized A. fumigatus alpha-mutant phytase.

A DNA sequence that encodes a phytase comprising an amino acid sequence that is at least 96.08%; or at least 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to the optimized A. fumigatus alpha-mutant phytase.

A DNA sequence which encodes a phytase and which (i) is at least 95.63%; or at least 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical; or (ii) hybridizes under low, or medium, medium/high, high, very high stringency conditions to
5 nucleotides 1-1401 of the DNA sequence encoding the optimized A. fumigatus alpha-mutant phytase.

A suitable negative control is DNA encoding A. fumigatus ATCC 13073 phytase. A suitable positive control is DNA encoding any one of the A. fumigatus ATCC 13073 alpha mutant phytase of
10 the optimised alpha-mutant.

(H) Phytases and corresponding DNA sequences related to consensus phytase-7

A phytase that comprises an amino acid sequence which is
15 at least 94.87%; or at least 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino acids 1-467 of consensus phytase-7 as shown in Fig. 10.

A phytase that comprises an amino acid sequence which is at least 95.30%; or at least 95.5, 96, 96.5, 97, 97.5, 98, 98.5,
20 99, 99.5% similar to the sequence of amino acids 1-467 of consensus phytase-7 as shown in Fig. 10.

A phytase which is encoded by a DNA sequence which is at least 96.38%; or 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to nucleotides 12-1412 of the DNA sequence of consensus phytase-
25 7 shown in Fig. 10.

A DNA sequence which encodes a phytase and which (i) is at least 96.38%; or at least 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical; or (ii) hybridizes under low, or medium, medium/high, high, or very high stringency conditions to nucleotides 12-1412
30 of the DNA sequence of consensus phytase-7 as shown in Fig. 10.

A DNA sequence which encodes a phytase comprising an amino acid sequence which is at least 94.87%; or at least 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino acids 1-467 of consensus phytase-7 as shown in
5   Fig. 10.

(I) Phytases related to basidio consensus phytase

A phytase which comprises an amino acid sequence which is at least 76.23%; or at least 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 94.5, 95, 95.5, 96, 96.5,
10   97, 97.5, 98, 98.5, 99, 99.5% identical to the combined sequence of (i) amino acids 1-441 of basidio consensus phytase shown in Fig. 3, and (ii) amino acids 1-26 shown in Fig. 5 (the sequence of (ii) to be added at the N-terminus of the sequence of (i)).

A phytase that comprises an amino acid sequence which is
15   at least 79.50%; or at least 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% similar to the sequence of amino acids 1-441 of basidio consensus phytase as shown in Fig. 3.

(J) Phytases related to consensus phytase-12

20   A phytase which comprises an amino acid sequence which is at least 70, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% identical to the sequence of amino acids 1-467 of consensus phytase-12 as shown in Fig. 21.

25   A phytase which comprises an amino acid sequence which is at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99, 99.5% similar to the sequence of amino acids 1-467 of consensus phytase-12 as shown in Fig. 21.

Table 8

Comparison of phytase amino acid sequences

Phytase	CP10	CP10-thermo[3]-Q50T-K91A	CP1-thermo[8]	CP1-thermo[8]-Q50T-K91A	CP11	CP7	Basidio	A. fumigatus alpha-mutant	A. fumigatus alpha-mutant (opt.)
Consensus phytase-1	95.08/93.79	94.65/93.36	98.50/98.29	98.07/97.86	92.06/90.70	95.29/94.86	69.42/62.16	85.59/82.58	84.73/81.72
A. niger NRRL3135	79.48/76.46	79.05/76.03	80.35/77.75	79.91/77.32	79.27/76.31	84.02/81.64	67.19/59.32	74.07/70.11	74.95/70/99
A. terreus 9-A1	76.04/72.11	75.82/71.90	76.47/72.33	76.25/72.11	76.51/73.02	75.76/71.18	65.39/58.02	69.67/64.84	69.45/64.84
A. terreus cbs	79.04/75.11	78.82/74.89	79.48/75.76	79.26/75.55	77.19/73.27	79.17/75.00	66.92/59.65	72.59/67.76	72.37/67.76
E. nidulans	78.70/74.35	78.26/73.91	79.78/75.87	79.35/75.44	80.56/76.62	76.96/73.04	67.20/58.13	72.39/67.83	72.11/67.54
A. fumigatus 13073	82.93/80.31	82.50/79.87	82.31/79.04	81.88/78.60	81.36/78.64	80.13/76.20	63.54/57.91	97.82/97.16	96.73/96.07
A. fumigatus 32239	81.30/77.39	80.87/76.96	81.09/77.61	80.65/77.17	79.95/76.08	79.13/75.22	63.61/54.97	90.22/86.52	89.57/85.87
T. themophilus	77.83/73.84	77.38/73.39	78.67/74.89	78.22/74.44	78.47/74.76	76.51/73.15	61.54/54.36	72.01/66.82	72.69/67.49
M. thermophila	69.16/62.81	69.48/63.33	69.27/62.84	69.59/63.36	69.65/63.06	68.82/62.13	65.56/57.91	66.21/58.45	66.44/58.68
T. lanuginosus	73.52/66.70	73.06/66.44	71.92/64.61	71.46/64.16	74.21/68.86	69.50/62.62	67.20/57.41	68.91/61.02	69.61/61.72
P. lycii	64.92/59.10	64.91/59.37	64.46/58.09	64.46/58.36	65.03/59.84	63.13/56.50	77.75/73.07	64.08/57.11	62.47/55.91
A. pediades	64.51/51.81	64.86/51.94	62.98/51.41	63.33/51.54	64.50/52.30	63.05/51.15	78.92/74.71	61.64/52.38	62.13/53.07
P. involutus 1	66.67/58.07	66.67/58.33	64.84/56.51	64.84/56.77	63.30/54.52	65.33/56.53	79.49/76.22	59.59/51.81	59.95/52.20
P. involutus 2	65.54/55.70	65.30/55.53	66.85/56.87	66.58/56.68	66.30/56.35	64.27/54.13	78.09/74.59	61.26/52.62	61.04/52.47

	T. pubescens	CP10	CP10t[3]Q50TK91A	CP1thermo[8]	CP1t[8]Q50TK91A	CP11	CP7	Basidio	A.fumigatus alpha-mut.	A. fum alpha-mut - opt.
T. pubescens	-	65.46/57.22	65.72/57.47	62.89/55.67	63.14/55.93	65.03/57.65	63.28/56.51	78.34/75.12	64.08/57.11	62.30/55.24
CP10	65.46/57.22	-	99.57/99.57	96.57/95.50	96.15/95.08	95.02/94.56	91.01/89.29	70.22/62.28	85.13/82.76	85.99/83.62
CP10t[3]Q50TK91A	65.72/57.47	99.57/99.57	-	96.15/95.08	96.57/95.50	94.56/94.10	90.58/88.87	70.47/62.28	85.13/82.76	85.99/83.62
CP1thermo[8]	62.89/55.67	96.57/95.50	96.15/95.08	-	99.57/99.57	93.42/92.29	94.43/93.79	68.40/60.74	84.52/81.94	85.38/82.80
CP1t[8]Q50TK91A	63.14/55.93	96.15/95.08	96.57/95.50	99.57/99.57	-	92.97/91.84	94.00/93.36	68.64/60.74	84.52/81.94	85.38/82.80
CP11	65.03/57.65	95.02/94.56	94.56/94.10	93.42/92.29	92.97/91.84	-	88.44/86.62	68.27/59.73	82.23/79.73	83.37/80.87
CP7	63.28/56.51	91.01/89.29	90.58/88.87	94.43/93.79	94.00/93.36	88.44/86.62	-	69.80/62.69	81.94/78.71	81.72/78.50
Basidio	78.34/75.12	70.22/62.28	70.47/62.28	68.40/60.74	68.64/60.74	68.27/59.73	69.80/62.69	-	65.97/60.52	66.41/60.68
A.fumigatus alpha-mut.	64.08/57.11	85.13/82.76	85.13/82.76	84.52/81.94	84.52/81.94	82.23/79.73	81.94/78.71	65.97/60.52	-	98.93/98.93
A. fum alpha-mut - opt.	62.30/55.24	85.99/83.62	85.99/83.62	85.38/82.80	85.38/82.80	83.37/80.87	81.72/78.50	66.41/60.68	98.93/98.93	-

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Table 9

Comparison of phytase encoding DNA sequences

Phytase	CP10	CP10-thermo[3]-Q50T-K91A	CP1-thermo[8]	CP1-thermo[8]-Q50T-K91A	CP7	Basidio	A. fumigatus alpha-mutant	A. fumigatus alpha-mutant (opt.)
Consensus phytase-1	95.87	95.87	98.72	98.36	96.37	65.46	66.88	66.88
A. niger NRRL3135	65.10	64.82	66.10	65.74	67.52	50.68	65.88	66.17
A. terreus 9-A1	61.74	61.53	62.17	62.03	60.53	49.40	66.24	66.31
A. terreus cbs	62.52	62.30	63.02	62.88	61.45	49.74	68.17	68.24
E. nidulans	65.08	64.94	65.30	65.01	64.22	49.92	64.90	65.44
A. fumigatus 13073	65.66	65.38	64.19	64.08	63.65	48.27	96.12	95.62
T. thermophilus	62.52	62.50	62.53	62.66	62.00	52.19	61.77	61.92
M. thermophila	55.51	55.15	55.36	55.22	53.91	48.44	58.17	58.24
T. lanuginosus	57.56	57.20	56.76	56.47	62.00	44.66	59.71	60.07
P. lycii	45.76	46.51	45.14	55.21	55.46	58.50	48.91	49.44
A. pediades	49.89	49.89	49.89	50.11	45.54	61.66	47.49	47.56
P. involutus 1	48.32	49.03	47.81	47.96	49.59	59.80	49.96	50.19
P. involutus 2	48.24	49.00	48.08	48.63	47.94	60.16	47.56	47.63
T. pubescens	47.00	47.17	46.46	47.62	46.83	60.37	49.89	49.96
CP10	-	99.43	96.40	96.05	93.73	66.40	67.81	68.24

CP10t[3]Q50TK91A	99.43	-	96.37	96.58	93.45	66.29	67.81	68.24
Cp1thermo[8]	96.40	96.37	-	99.65	95.30	65.40	66.74	67.17
CP1t[8]Q50TK91A	96.05	96.58	99.65	-	94.94	65.47	66.74	67.17
CP7	93.73	93.45	95.30	94.94	-	64.56	65.88	65.88
Basidio	66.40	66.29	65.40	65.47	64.56	-	50.41	50.49
A.fumigatus alpha-mut.	67.81	67.81	66.74	66.74	65.88	50.41	-	99.50
A. fum alpha-mut opt.	68.24	68.24	67.17	67.17	65.88	50.49	99.50	-